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THE EFFECT IN VITRO OF GAMMA INTERFERON ON CELL PROLIFERATION, COLLAGEN PRODUCTION AND PROCOLLAGEN mRNA EXPRESSION IN HYPERTROPHIC SCAR FIBROBLASTS

by

ALAN ROBERTSON HARROP

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **MASTER OF SCIENCE**

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

Fall, 1991

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QUOTATION

YOU WON'T KNOW A THING

UNTIL YOU GET INSIDE.

- J.D. Morrison

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **THE EFFECT IN VITRO OF GAMMA INTERFERON ON CELL PROLIFERATION, COLLAGEN PRODUCTION, AND PROCOLLAGEN mRNA EXPRESSION IN HYPERTROPHIC SCAR FIBROBLASTS**

submitted by **ALAN ROBERTSON HARROP**

in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **EXPERIMENTAL SURGERY**

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Date: October 4 1991

ABSTRACT

Hypertrophic scarring (HTS) is an abnormal form of wound healing characterized by an overaccumulation of collagen. Clinically, the condition presents as a red, raised, nodular scar which, in comparison to normal scars, undergoes a prolonged and incomplete regression. Extensive HTS, as is often seen following thermal injury, may be both functionally and cosmetically debilitating. Current modalities of treatment include pressure garments, silicone gel sheets, intralesional steroid injections and surgical excision with resurfacing. Unfortunately these are often inadequate for controlling the problem.

The pathogenesis of HTS is incompletely understood however its development and resolution may be influenced by various cytokines. This study looks at the effect of the cytokine gamma interferon (IFN) on HTS fibroblasts in vitro.

Fibroblast cultures were established from 5 thermally injured patients with HTS. In addition cultures were established from site-matched unaffected normal skin in the same patients. Normal and HTS fibroblasts from each of these cultures were then treated with varying doses of IFN and compared to untreated cells. Specific responses examined were: rate of proliferation (by serial cell counts), collagen production (by mass spectrometric analysis of hydroxyproline released into the culture medium) and types I and III procollagen mRNA levels (by Northern blot analysis).

Treatment with 1000 U/mL IFN for 5 days reduced cell proliferation in HTS and normal cells to 49% and 65 % of the untreated states, respectively ($p<0.05$ for each). Collagen production was reduced to 66%

and 75% of the untreated states in HTS and normal cells, respectively ($p<0.05$ for each). Following treatment for 12 hours with IFN, types I and III procollagen mRNA levels were reduced to 45% and 64% of the untreated state, respectively, in HTS cells ($p<0.05$) and 50% and 62% of the untreated state, respectively, in normal cells ($p<0.05$).

We conclude that gamma IFN has an inhibitory effect on cell proliferation and collagen production in both normal and HTS fibroblasts. Furthermore, the latter response would appear to be a result of reduced procollagen mRNA levels. These findings support the potential role for gamma IFN as a down regulatory growth factor for the treatment of HTS following burn injury.

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LIST OF ABBREVIATIONS

DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
GC/MS	Gas Chromatography/Mass Spectrometry
Gly	Glycine
HTS	Hypertrophic Scar
Hyp	Hydroxyproline
IFN	Interferon
Lys	Lysine
mRNA	Messenger Ribonucleic Acid
O_2	Oxygen
Pro	Proline
RNA	Ribonucleic Acid

CHAPTER 1

INTRODUCTION

It is a remarkable fact of life that tissue injury is usually succeeded by some form of wound healing. This takes the form of either regeneration with replacement of injured tissue by parenchymal cells of the same type or, more commonly, replacement by a connective tissue stroma otherwise known as a scar [1]. It is an unfortunate paradox that the latter process is sometimes flawed by excessiveness. Examples of this include hepatic cirrhosis, pulmonary fibrosis and dermal fibrosis [2,3].

One form of dermal fibrosis familiar to surgeons is hypertrophic scarring. This is a process that may occur consequent to any traumatic injury extending into the dermis of the skin [4]. It develops frequently following burn injuries but also occurs at sites of surgical incision or other penetrating trauma. A precise clinical definition of what constitutes a hypertrophic scar is elusive with the distinction between hypertrophic and normal scars being somewhat arbitrary. Hunt [5] provides a practical definition when he describes the hypertrophic scar as a "scar with more mass and less flexibility than the surgeon or patient wishes to tolerate." On a microscopic level, this is a condition of overaccumulation of collagen within the healing wound due, at least in part, to excessive production of this extracellular matrix protein by mesenchymal cells called fibroblasts [6].

The challenge facing clinicians and scientists dealing with disorders of fibrosis such as hypertrophic scarring is not only to better understand why they occur but also to develop therapeutic strategies to modulate the fibroproliferative response.

A multitude of growth factors are believed to contribute to the wound healing process and are also felt to play a role in the development of fibroproliferative disorders [2,5,7,8]. There is currently a great deal of research directed at understanding these interactions.

Gamma interferon is one such cytokine suggested to play a regulatory role in wound healing. It is produced naturally by activated T lymphocytes and has been shown to reduce collagen production by normal fibroblasts in culture [9]. There is thus merit to its consideration as a possible therapeutic agent for the control of fibroproliferative disorders.

The focus of interest in this thesis is the effect of gamma interferon on cultured hypertrophic scar fibroblasts obtained from patients with hypertrophic scarring as a result of burn injuries. Hypertrophic scarring is particularly problematic in such patients because they often have extensive areas of injury, causing both cosmetic and functional morbidity.

Chapter 2 is a review of the topic of collagen metabolism. It is necessary to understand the normal aspects of collagen structure, biosynthesis and degradation before looking at abnormalities.

Chapter 3 is a review of the clinical aspects of hypertrophic scarring, the specific fibroproliferative disorder under study. Included are discussions regarding its clinical features, etiology, possible pathogenesis as well as current methods of treatment.

Chapter 4 reviews the work to date looking at the effect of gamma interferon on normal fibroblasts and fibroblasts that overproduce collagen thus providing the impetus for studying its effect on hypertrophic scar fibroblasts.

Chapter 5 is the experimental component of the thesis. Specifically examined is the effect of gamma interferon on cell proliferation, collagen production and procollagen mRNA expression by hypertrophic scar fibroblasts from 5 patients as compared to control fibroblasts from unaffected areas in the same patients.

It is hoped that this work will contribute not only to the understanding of hypertrophic scarring but perhaps also to the treatment of this debilitating condition.

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CHAPTER 2

OVERVIEW OF COLLAGEN METABOLISM

STRUCTURE OF COLLAGEN

The mammalian extracellular matrix is composed of two fibrous proteins, elastin and collagen, surrounded by proteoglycans [1]. Collagen plays an important role in the provision of strength and integrity for tissues throughout the body. In addition, the process of repair in all tissues depends on collagen production and its subsequent modifications [2]. The architectural development and function of the various organs throughout the body are dependent upon the heterogeneity of collagen and proteoglycans, as well as the relative ratio of the matrix components. Derangement of collagen metabolism may result in either overaccumulation or underaccumulation of collagen within the matrix [1]. An understanding of normal collagen structure, biosynthesis and degradation is thus helpful in understanding states of abnormal collagen metabolism and in studying potential means of regulation.

Collagen molecules are long molecules consisting of a triple helix of three polypeptide chains, each referred to as an alpha chain [3]. There are several different types of alpha chain which when variously combined in a triple helix, allow for the existence of at least 12 different types of collagen [4]. The individual alpha chains are left-handed helices which in turn are wrapped around each other in a right-handed triple super-helix [5].

While each type of alpha chain is a unique gene product with a unique amino acid sequence, there are many similarities in these chains. All of the alpha chains have at least one domain consisting of a repeating triplet of glycine and two other amino acids. This is represented by the formula $(Gly\text{-}X\text{-}Y)_n$. In types I, II, and III collagen, n is approximately 334, while in type IV collagen, n is approximately 490 [3]. There is an absolute requirement in the triple helical domain for glycine in every third position as it has no side chain and is thus small enough not to disrupt the helix. The side chains of the amino acids in the X and Y positions are directed outward where they are free to participate in intermolecular and intramolecular interactions [5]. The Y position is often occupied by 4-hydroxyproline. The amino acids proline and hydroxyproline constitute 20-25% of all residues in a given alpha chain [3]. For example, a 1000 residue alpha 1 (I) chain, which contributes to the formation of type I collagen, contains 97 hydroxyproline residues and 129 proline residues. An alpha 2 (I) chain, also contributing to type I collagen, contains 86 hydroxyproline residues and 113 proline residues per 1000 residues. Finally, an alpha 1 (III) chain which contributes to type III collagen contains 121 hydroxyproline residues and 102 proline residues per 1000 residues [3].

Some of the lysine residues present are hydroxylated and some of the resulting hydroxylysine residues are glycosylated with galactose or glucosylgalactose [3].

The triple helix structure is stabilized in several ways [5]:

1) Hydrogen bonds - between glycine and the residue in the Y position in a neighboring chain

2) Ring structure of proline - prevents rotation at the N-C alpha bond of the polypeptide chain

3) Hydroxyproline - contributes water-bridged hydrogen bonds

4) Glycine - small size permits close packing of the triple helix

5) Side-chain interactions - including hydrogen bonds, charged-pair interactions and hydrophobic interactions

6) Interchain disulfide bonds - present in type III and type IV collagen

The alpha chains are synthesized initially as larger pro-alpha molecules which are longer at the N-terminal end by about 150 amino acids and at the C-terminal end by about 250 amino acids. These regions are called the N- and C-terminal propeptides, respectively. Within the cell, 3 pro-alpha chains are assembled to form a procollagen triple helix which is in turn secreted from the cell. After secretion the N- and C-terminal propeptides are cleaved from the procollagen leaving a tropocollagen molecule, the basic structural unit of collagen [5].

As mentioned, there are several different types of collagen. The collagen type depends on the types of alpha chain present in the helix [4]:

Type I - $[\text{alpha 1 (I)}]_2$ [alpha 2 (I)]

Type II - $[\text{alpha (II)}]_3$

Type III - $[\text{alpha (III)}]_3$

Type IV - $[\text{alpha 1 (IV)}]_2$ [alpha 2 (IV)]

Type V - [alpha 1 (V)] [alpha 2 (V)] [alpha 3 (V)]

Type VI - [alpha 1 (VI)] [alpha 2 (VI)] [alpha 3(VI)]

Type VII - $[\text{alpha (VII)}]_3$

Type VIII - $[\text{alpha (VIII)}]_3$

Type IX - [alpha 1 (IX)] [alpha 2 (IX)] [alpha 3 (IX)]

Type X - $[\text{alpha (X)}]_3$

Type XI - [alpha 1 (XI)] [alpha 2 (XI)] [alpha 3 (XI)]

Type XII - $[\text{alpha (XII)}]_3$

After cleavage of the N- and C-terminal propeptides, the resulting tropocollagen molecules are assembled systematically to form collagen fibrils. Adjacent molecules of tropocollagen are staggered by 67 nm (slightly less than one-fourth the length of a single molecule). This creates a 67 nm periodic pattern seen by electron microscopy [4].

TISSUE DISTRIBUTION OF COLLAGEN [4]

Type I collagen, which has a relatively low hydroxylysine content and few sites of glycosylation is distributed widely throughout the body. It is prominent in skin, tendon, bone, dentin and fascia. Type II collagen, which is rich in hydroxylysine and is heavily glycosylated is found in cartilage, notochord and vitreous body. Type III collagen has a high hydroxyproline content, low hydroxylysine content, interchain disulfide bonds between cysteines at the carboxy-end of the helix and few sites of glycosylation; it is found in most tissues which also contain type I collagen, examples being skin, uterus and blood vessels. Type IV collagen has a high hydroxylysine content, is heavily glycosylated, is relatively rich in 3-hydroxyproline and has a low alanine content. It is present in lens capsule and basement membranes of epithelial and endothelial cells. Type V collagen is high in hydroxylysine, is heavily glycosylated and has a low alanine content; it is distributed throughout the exoskeleton of fibroblasts and other mesenchymal cells and is also found in the basement membrane of smooth and striated muscle cells.

Human skin is particularly rich in collagen. Of this 80% is type I, 15% is type III and the remaining 5% consists of type I trimer, type IV, type V, type VI, and type VII [6].

Hydroxyproline, one of the constituent amino acids of collagen is of special interest. Small amounts of hydroxyproline are found in elastin, the Clq component of the complement system and also the collagenous tail of the acetylcholinesterase enzyme. Aside from these few exceptions however, hydroxyproline in vertebrate tissues is unique

to collagen [5]. As well, since eukaryotic cells have no transfer RNA for hydroxyproline, free hydroxyproline cannot be incorporated into newly synthesized polypeptides. Finally, since hydroxyproline cannot be reutilized, its excretion is an indirect measure of catabolism of collagen [3].

COLLAGEN BIOSYNTHESIS

a. Transcription

Each alpha procollagen chain is coded for by a distinct gene. The genes include exons (expressed) and introns (not expressed) and therefore are much longer than the corresponding translatable mRNA [5]. The gene is transcribed into a precursor mRNA with representation from both the introns and exons. This precursor is in turn spliced to form cytoplasmic translatable mRNA [5].

b. Translation

The mRNA is translated to yield procollagen alpha chains. This occurs on ribosomes on the membrane of the rough endoplasmic reticulum (RER). The initially synthesized polypeptide chain has a hydrophobic signal sequence on the N-terminal which binds to the membrane of the RER and leads the nascent polypeptide chain across the membrane into the cisternae of the RER [5]. Soon thereafter the signal peptide is cleaved from the rest of the molecule.

c. Post-translational Modifications

Within the RER, hydroxylation of proline and lysine residues occurs catalyzed by peptidyl prolyl 4-hydroxylase, peptidyl prolyl 3-hydroxylase and peptidyl lysyl hydroxylase. In each case Fe++, 2-oxoglutarate, O_2 and ascorbic acid are required in the reaction [5]. Hydroxylation begins while the chain is still growing on the ribosome and continues after release of the chain from the ribosome. In general, peptidyl prolyl 4-hydroxylase only acts on prolyl residues in the X-Pro-Gly sequence and peptidyl lysyl hydroxylase only acts on lysyl residues in the X-Lys-Gly sequence. Peptidyl prolyl 3-hydroxylase generally only acts on proline in the Pro-4Hyp-Gly sequence [5]. The triple helix conformation prevents all three hydroxylations. 4-hydroxyproline is felt to be important in stabilizing the triple helix [6]. The hydroxy-group of hydroxylysine has two functions: it acts as a site of attachment for carbohydrate units and it is involved in intermolecular collagen crosslinks [5]. The function of 3-hydroxyproline is not known.

A second modification of the alpha chain involves glycosylation of hydroxylysine residues. Here, galactose is transferred to hydroxylysine, catalyzed by the enzyme galactosyl-transferase. Some of the resulting galactosylhydroxylysine residues are then further glycosylated by the addition of glucose catalyzed by the enzyme glucosyl-transferase [3]. The usual carbohydrate donor for these glycosylations is the corresponding uridine diphosphate (UDP) glycoside. Both reactions require a bivalent cation, preferably manganese [5]. Glycosylation requires that lysine has been hydroxylated and is limited by formation of the triple helix [3]. It is felt that glycosylation

occurs while the polypeptide is still being produced on the ribosome attached to the RER [3,5]. The exact role of the glycosylated hydroxylysine residues is not known; however, it may be important in the packing of collagen molecules into larger fibrils [5], and may in some way contribute to the interaction of collagen with other components of the extracellular matrix and with cell surfaces [3].

Once translation is complete, numerous inter- and intrachain bonds are formed involving the propeptides of the alpha chains. Interchain disulfide bonds are found in types I and II procollagen only between the C-terminal propeptides, whereas in type III collagen interchain disulfide bonds are found between both the C- and N-terminal propeptides. Type III also contains two interchain disulfide bonds at the C-terminal end of the triple helical region of the collagen domain [5].

The interchain disulfide bonds between the C-terminal propeptides seem to be a starting point for triple helix formation. Helix formation takes place either in the cisternae of the RER after release of the pro alpha chain into it or within the Golgi apparatus [5]. Helix formation is a spontaneous process.

d. Secretion of Procollagen

Within the Golgi apparatus procollagen molecules become aligned in dense packets in preparation for secretion from the cell. The Golgi secretion vacuoles are then moved to the surface of the cell for secretion by exocytosis. These processes require the assistance of the cellular microtubule system [3]. Inhibition of the microtubular system with colchicine or vinblastine results in the accumulation of procollagen within the Golgi apparatus [7,8].

e. Cleavage of the Propeptides

Once the procollagen has been secreted into the extracellular space, the propeptides which until this point have kept the molecule soluble, are removed. This cleavage is catalyzed by the enzymes procollagen N-peptidase and procollagen C-peptidase which remove the N- and C-terminal propeptides, respectively [3]. The cleaved propeptide sequences are believed to act as feedback regulators of cellular collagen synthesis [5].

f. Fibril Formation

Following cleavage of the propeptides the resulting collagen molecules spontaneously aggregate into fibrils [3]. The integrity of the fibrils is improved by the formation of crosslinks between the collagen molecules. Crosslinking involves two steps. Firstly, oxidative deamination of lysine and hydroxylysine residues by the enzyme peptidyl lysine oxidase, produces reactive aldehydes. This reaction requires copper as a cofactor. These aldehydes in turn participate in

the formation of cross-links between molecules, predominantly Schiff bases which may rearrange to ketoamines [5].

DEGRADATION OF COLLAGEN

Degradation of collagen may occur in several ways. Firstly, there may be intracellular degradation of new procollagen prior to secretion. Secondly, within the extracellular space there may be degradation of collagen by collagenase. Finally, there may be phagocytosis of extracellular collagen and subsequent intracellular degradation. Collagen degradation by whatever mechanism serves to offset accumulation by new collagen production.

a. Intracellular Degradation of Procollagen

Several investigators have presented evidence for intracellular degradation of new procollagen, both in vitro [9,10,11] and in vivo [12,13]. Various studies suggest that 10-40% of newly synthesized collagen is degraded within the cell [14]. Experiments in which extracellular degradation is blocked with extracellular protease inhibitors suggest that the two processes may occur simultaneously but that they occur independently of one another [15].

The purpose of intracellular degradation is suspected to be twofold. Firstly, it may be a mechanism of destruction of abnormal collagen as suggested by experiments in which proline analogues are incorporated into new alpha chains which in turn cannot form a stable triple helix. In this situation an increase in intracellular degradation has been observed and is hypothesized to be degradation of the abnormal alpha chains [9]. Similarly, if hydroxylation of proline

is blocked (for example by the absence of ascorbic acid), an increase in intracellular degradation is observed [16]. Secondly, it has been suggested that intracellular degradation may be a means of regulating the rate of collagen production [14].

b. Extracellular Degradation

Extracellular degradation of collagen occurs through the activity of the enzyme collagenase. This enzyme makes a single break in each alpha chain of the triple helix at the site of a glycyl-isoleucine bond [17]. This in turn results in an unwinding or denaturation of the collagen molecule. The denatured collagen is then susceptible to degradation by other extracellular proteolytic enzymes [3].

c. Phagocytosis and Intracellular Degradation

Quantitative stereologic studies with electron microscopy have suggested a third type of collagen degradation in which the fibroblast phagocytoses fragments of collagen fibrils and subsequently degrades these within the cell [10,11].

POSSIBLE SITES OF REGULATION OF COLLAGEN METABOLISM

An understanding of collagen metabolism has important clinical implications. Numerous pathological conditions exist in which there is overaccumulation of collagen within the extracellular matrix. Examples of these include hypertrophic scarring, pulmonary fibrosis, hepatic cirrhosis and tendon adhesions. Regulation of collagen metabolism might be of therapeutic benefit in such conditions. From the previous discussion of collagen biosynthesis it is evident that regulation could

theoretically occur at many levels, including gene selection, transcription, messenger splicing, translation, hydroxylation of proline and lysine, glycosylation of hydroxylysine, glycosylation of propeptides, fibril formation, cross-linking of fibrils, intracellular degradation and extracellular degradation. Regulation of some of these processes in turn could potentially reduce excess collagen deposition.

While there would be great benefit from local and systemic measures for controlling excess collagen deposition, a potential trade-off, especially with systemic measures of control, is an adverse effect on normal collagen metabolism in other areas of the host [18].

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CHAPTER 3

HYPERTROPHIC SCARRING: CLINICAL FEATURES, ETIOLOGY, PATHOGENESIS AND CURRENT TREATMENT

INTRODUCTION

There exist many pathological conditions in which there is overaccumulation of collagen within an organ. Common examples of these include pulmonary fibrosis and hepatic cirrhosis. The skin may also be affected by fibrotic processes and dermal fibrosis is the hallmark of several inherited and acquired conditions [1]. Examples of dermal fibrosis include scleroderma (localized and systemic), bleomycin-induced fibrosis, keloids and hypertrophic scars. Hypertrophic scarring is of particular interest to surgeons, as it is a not uncommon sequela to traumatic injuries and wound healing.

THE USUAL PROCESS OF SCAR FORMATION

Following surgery or trauma causing injury to the deep dermis of the skin, a sequence of wound healing events occurs which ultimately result in the formation of a scar. Classically this occurs in three phases: inflammatory, fibroblastic and maturational [2]. The inflammatory phase immediately follows wounding and is characterized by increased blood flow to the area and increased capillary permeability. Fibrin clots and seals the wound. There is an accumulation of inflammatory cells within the wound which attempt to remove foreign or necrotic tissue. New epithelial cells quickly grow across the surface of the wound.

In the second phase (fibroblastic phase), fibroblasts move into the fibrin clot and begin to synthesize new collagen. This results in rapid strengthening of the wound. Adequate supplies of Vitamin C, zinc and protein precursors are required for this process.

The maturation phase is characterized by remodelling of the collagen within the wound by simultaneous processes of collagen production and degradation. Associated with this is a gradual increase in wound strength up to one year following the injury. Likewise the external appearance of the wound changes from a thick, red, raised and nodular scar one month following the injury to a softer, flatter, paler and smoother scar at six months to a year.

ABNORMAL SCAR FORMATION - THE CLINICAL PROBLEM

In certain cases the above sequence of events goes awry and wounds heal with large, raised collagenous scars known as keloids or hypertrophic scars. The respective clinical courses and physical characteristics define hypertrophic scars and keloids as separate entities. For unknown reasons keloid and hypertrophic scarring appear to be unique to man and thus no good *in vivo* animal model exists [3].

Hypertrophic scars are red, raised, nodular scars which remain within the boundaries of the original wound. In contrast to normal scars these characteristics undergo a prolonged and incomplete regression [4]. Hypertrophic scars are prone to recurrence following surgical excision. The incidence of hypertrophic scarring is higher in patients with darkly pigmented skin (for example Negroes and North

American Indians). Younger patients (particularly children) are more likely to form hypertrophic scars than older patients [2].

Keloid scars are also red, raised, nodular scars, however they always overgrow the confines of the original wound [4]. There is a high likelihood of recurrence after excision, often with a worse appearance than the original lesion. As with hypertrophic scars, keloids occur more frequently in patients with dark pigmentation and in younger patients. Keloids may occur anywhere but are frequently found over the shoulders, upper back, anterior chest and upper arms. Less frequent locations are the lower extremities, face and neck. Rarely they may occur on eyelids, genitalia, palms, soles, cornea and mucous membranes [5].

Both hypertrophic scars and keloids may be problematic in the clinical setting. They may be cosmetically unacceptable and may also become a functional problem by limiting mobility of joints and areas of normally loose skin or by narrowing body orifices.

It is generally not possible histologically to distinguish between keloids and hypertrophic scars. Both are more vascular and contain a higher number of fibroblasts than either normal skin or normal scar [6]. In addition, both often display swirl-like perivascular clusters of fibroblasts [5]. Electron microscopy reveals that the collagen bundles in hypertrophic scar and keloid lie in a haphazard fashion in contrast to normal scar where the bundles lie parallel to the epithelial surface in discrete groups [5,6].

INCITING FACTORS FOR KELOID AND HYPERTROPHIC SCARRING

Keloid and hypertrophic scarring are known to occur following a large variety of skin injuries. These include surgical incisions, lacerations, tattoos, burns, injections, bites, vaccinations and ear piercing [5]. In some cases of keloid, however, patients cannot recall any history of preceding trauma. It is not known why, given the same injury, some patients develop abnormal scarring while others do not.

POSSIBLE ETIOLOGY OF HYPERTROPHIC SCARRING AND KELOID

The precise etiology of these forms of abnormal scar formation is not known however several theories have been put forth as are outlined below.

a. Skin Tension

Skin tension may contribute to abnormal scarring as evidenced by the fact that both keloid and hypertrophic scar occur frequently in areas of increased tension, such as the presternal and deltoid regions [7]. Supporting this is a study by Calnan and Copenhagen [8] in which excised keloid was autografted onto an area of relatively little tension and was noted in all cases to undergo some degree of macroscopic atrophy. Nevertheless, keloids sometimes occur in areas of low skin tension. The earlobes for example, which are free of tension, develop keloid relatively commonly [6].

b. Genetic Factors

Both keloid and hypertrophic scarring are more common in patients with other affected family members [3,9]. A specific genetic mode of inheritance, however, has not been determined. There does not seem to be a relation between the HLA A and B loci and hypertrophic scarring [10]. In a small study of 19 patients however, there has been found to be an association between the HLA-DR beta 16 allogenotype and hypertrophic scar formation [11]

c. Endocrine Factors

There has been speculation that endocrine factors may play a role based on the clinical observations that keloids often enlarge at puberty and during pregnancy. Keloids have also been noted occasionally to decrease in size following menopause [5].

d. Tissue Hypoxia

Some investigators feel that local tissue hypoxia may contribute to the formation of keloid and hypertrophic scar. Sloan and colleagues [12] performed mass spectrometric measurements of tissue gases obtained from probes placed into hypertrophic scar and normal dermis in burn patients. It was found that the pO_2 level in hypertrophic scar was significantly depressed in comparison to normal dermis. Hunt and associates [13] found increased tissue lactate levels in healing animal wounds. Increased lactate levels are in turn felt to increase collagen production by fibroblasts [13,14]. In an attempt to understand how tissue hypoxia is established in a wound, Kischer and colleagues [15] used electron microscopy to study the microvasculature of keloid and

hypertrophic scar tissue. They found increased occlusion of the microvessels by endothelial cell proliferation and myofibroblast contraction in the abnormal scars when compared to normal dermis. They believe that microvascular occlusion in turn causes tissue hypoxia and anaerobic metabolism which stimulates excessive collagen production.

PATHOGENESIS OF HYPERTROPHIC SCAR FORMATION

While the etiology of hypertrophic scarring is not clear, some progress has been made towards elucidating the pathogenesis of the problem. Since the basic underlying problem seems to be overabundant deposition of collagen, various investigators have looked at the different components of collagen biosynthesis in the hypertrophic scar. Relatively more studies have addressed these issues with respect to the keloid problem; however these may have relevance to hypertrophic scarring. In general, these studies are *in vitro* studies since no animal is known to develop true hypertrophic or keloid scars.

a. Fibroblast Growth Kinetics

Several studies have looked at the growth kinetics of normal dermal fibroblasts, hypertrophic scar fibroblasts and keloid fibroblasts in culture. One group [16], looking at fibroblast growth kinetics using continuous [^{3}H]-thymidine labelling, has observed a shorter DNA synthesis time but reduced growth fraction and labelling index in hypertrophic scar-derived fibroblasts compared to normal fibroblasts; this was interpreted to suggest that in hypertrophic scars most fibroblasts are dormant although a small number of fibroblasts proliferate more actively than normal fibroblasts. Most other

investigators however have shown no significant difference in the doubling times of the different cell strains [17,18,19]. Of note, these studies looked at fibroblasts from different patients. There have been no studies of growth kinetics using fibroblasts from normal dermis and hypertrophic scar in the same patient.

b. Enzyme Activity

Other investigators have studied the activity of some of the enzymes associated with collagen biosynthesis. Specifically, the enzyme prolyl hydroxylase has been noted to be increased in keloid and hypertrophic scar as compared to normal skin [20,21]. Prolyl hydroxylase is required for hydroxylation of proline during collagen biosynthesis and the above finding is indirect evidence of collagen overproduction.

c. Collagen Synthesis

More recently, attention has been focused on more direct analysis of collagen synthesis by fibroblasts from hypertrophic scar. One study looked at fibroblasts from normal human skin, normal and hypertrophic scar and keloid in a tissue culture environment. Here, rates of collagen synthesis were determined by measuring the rate of incorporation of [^{14}C]-proline into peptide-bound [^{14}C]-hydroxyproline. These investigators observed increased collagen synthesis in keloid and hypertrophic scar in comparison to normal scar; however the rates of collagen synthesis in hypertrophic scar and keloid fibroblasts were similar to that of normal skin [22]. Another study, using cultured fibroblasts from normal skin, normal scar and keloid, looked at collagen

synthesis as represented by incorporation of [^{3}H]-L-5-proline into peptide-bound [^{3}H]-hydroxyproline. The fibroblasts were subcultured at least four times, thus eliminating the possibility that any in vivo, exogenous, collagen-stimulating factor (local or systemic) would still be present. Keloid fibroblasts were seen to synthesize two to three times more collagen per cell than either normal skin or normal scar, while growth kinetics were the same [17]. Again, neither of these reports involved studying normal and abnormal fibroblasts from the same patient.

On the other hand, there exist studies which demonstrate a different picture. Ala-Kokka and colleagues [23], obtained keloid biopsies from 9 patients and normal dermis control biopsies from 8 sex- and age-matched patients. A ninth control biopsy was obtained from normal looking skin in one of the keloid patients. Part of each specimen was analyzed for collagen concentration and there was found to be no significant difference between the two groups. The activities of prolyl 4-hydroxylase and galactosylhydroxylysyl glucosyltransferase (enzymes participating in intracellular collagen biosynthesis) were however significantly elevated in the keloid tissue, indirectly suggesting increased collagen production. Another part of each specimen was used to grow fibroblast cell cultures. In turn, these fibroblasts were examined for collagen synthesis by looking at [^{14}C]-proline incorporation into peptide-bound [^{14}C]-hydroxyproline. The mean collagen synthesis rates did not differ between the two groups, however one keloid cell strain exhibited a higher rate than the mean plus two standard deviations of the control group. As well, in the one set of

cultures obtained from keloid and normal skin in the same patient the rate of collagen synthesis was higher in the keloid fibroblasts. When total RNA was examined by Northern blot analysis and slot blot hybridization, no significant difference in mean mRNA levels was noted between the two groups, however when looking at the set of fibroblasts from keloid and normal skin in the same patient, mRNA levels were higher in the keloid fibroblasts.

Similarly, Russell and colleagues [24] compared collagen biosynthesis in normal and keloid fibroblasts from different patients by looking at [^{3}H]-proline incorporation into collagen. In this experiment they noted no difference in collagen or non-collagen protein production. They did however note that physiologic concentrations of hydrocortisone inhibited collagen production in normal fibroblasts by 60% but not at all in keloid fibroblasts.

In a related study by the same group [25], addition of physiologic concentrations of hydrocortisone resulted in reduced collagen production and procollagen mRNA levels in the normal fibroblasts but no change in these parameters in the keloid fibroblasts. This was interpreted to suggest that the keloid fibroblasts may respond differently to the body's usual regulatory mechanisms.

Finally, Abergal and colleagues [26] examined 9 different keloid fibroblast strains for collagen synthesis as determined by incorporation of labelled proline. These were compared to fibroblasts from normal site-matched skin in age-matched people. Of these keloid fibroblasts, 4 strains synthesized normal amounts of collagen, 1 showed slightly

increased collagen production and 4 strains were clearly overproducers of collagen.

Thus, it would seem that in the in vitro situation, some but not all keloid and hypertrophic scar fibroblasts are overproducers of collagen. The reason for these discrepancies is not clear but could be related to a selection process for cells with different biosynthetic capacities either in the lesion itself or more likely during the establishment of the fibroblast cultures. It is also possible that the cells themselves are the same but exist in different milieus in vivo. Nonetheless, the culture environment does provide a useful system for the isolated study of factors possibly contributing to excessive collagen production and of possible methods of control.

d. Collagen Degradation

The possibility exists that altered collagen degradation contributes to keloid and hypertrophic scar formation. Studies in this area have yielded conflicting results. Tissue levels of collagenase activity have been studied in keloid and hypertrophic scar and found to be no greater than in normal dermis [27,28]. With fibroblasts grown in culture, one group [27] has shown increased collagenase activity in keloid fibroblasts in comparison to normal fibroblasts while another group [29] found no difference in collagenase activity amongst normal, hypertrophic scar and keloid fibroblasts.

In another study, cultured fibroblasts from keloid and normal skin were incubated with [^{14}C]-proline. Subsequently, protein was precipitated from the combined cell lysate and medium preparations by

the addition of cold trichloroacetic acid. There was no difference in the TCA-soluble [^{14}C]-hydroxyproline content suggesting that the rate of collagen degradation is the same for both [17].

Finally, increased concentrations of alpha-2-macroglobulin have been noted in keloids and HTS [30]. Both of these are known to be inhibitors of collagenase. Thus, these investigators hypothesize that elevated tissue concentrations of alpha-globulins may contribute to overaccumulation of collagen in keloids and HTS by inhibiting collagenase activity. These same investigators also showed that following intralesional injection of steroids there was both clinical improvement and reduced alpha-1-antitrypsin concentration in the scars. They believe that steroids reduce alpha-globulins activity in the scar which in turn increases collagenase activity.

Thus, there is a small amount of evidence that collagen degradation is altered in keloid and hypertrophic scar. It is likely however, that enhanced collagen synthesis is the major factor leading to excess deposition of collagen [6].

e. Abnormal Response to Cytokines

<u>In vivo</u> it has been observed that infiltrates of lymphocytes and monocytes are present in tissues undergoing both normal and pathological fibrosis, leading to the idea that abnormal responses to cytokines or abnormal production of cytokines may play a role in the development of pathological scarring, including keloid and hypertrophic scarring.

In fibroblast cultures from both normal dermis and scleroderma patients, human interleukin-1 enhanced collagen production as measured by incorporation of [^{3}H]-proline into collagen [31]. It seems possible that IL-1 may play a role in pathological states of fibrosis. Other investigators have noted that IL-1 stimulates fibroblast proliferation in culture [32].

Others have looked at topical application of various cytokines, alone or in combination, to partial-thickness skin wounds in pigs. Transforming growth factor alpha (TGF-alpha), produced by platelets, macrophages and a variety of other cells, resulted in increased collagen content of the wounds as determined by histological examination and quantitation of hydroxyproline content [33].

Tsutsumi and colleagues [34] looked at the effect of numerous cytokines on cultured fibroblasts and hepatocytes. They found that interleukin-1 alpha, interleukin-1 beta and transforming growth factor alpha increased collagen production by fibroblasts and hepatocytes in culture. Transforming growth factor beta increased collagen synthesis in fibroblasts but inhibited collagen synthesis in hepatocytes.

Other cytokines have been shown to inhibit collagen production by fibroblasts. Alpha interferon and gamma interferon for example have both been shown to decrease collagen production by cultured fibroblasts [34]. It has been suggested then that reduced levels or cell resistance to these inhibitory cytokines may contribute to states of fibrosis.

It thus seems possible that cytokines may play a role in the development of various abnormal states of fibrosis, including HTS, keloid, hepatic fibrosis, scleroderma and pulmonary fibrosis. It is

also evident however that many cytokines could be involved in a complex interconnected fashion.

f. Possible In Vivo Model for Studying Hypertrophic Scarring

Robb and colleagues [35] have proposed an in vivo model for looking at HTS. The model involves grafting of human hypertrophic scar or normal (control) scar to the bed of a full thickness wound on the back of a nude (athymic) mouse. Immunosuppression is not required since the nude mouse lacks a thymus gland and therefore is immunologically unable to reject the human tissue. In this study, normal skin, hypertrophic scar and normal scar all were accepted and revascularized by the recipient nude mouse for at least 4 months. The different tissues retained their original gross and microscopic characteristics. In another variation of the model, normal human skin was applied to the mouse and burned (causing a partial-thickness injury) at a later date. The area developed the gross and microscopic characteristics seen in humans following the healing of a partial-thickness burn. Black skin exhibited a greater degree of scarring than white skin, as is often seen in the clinical setting.

Kischer and colleagues [36] used a similar model in which pieces of hypertrophic scar tissue were implanted into subcutaneous pockets in nude mice where they were carried for varying periods of time up to 246 days. The implants were not rejected and retained their original histologic features regardless of the length of implantation, suggesting that this might be another in vivo model for the study of hypertrophic scarring or treatments directed towards its resolution.

CURRENT METHODS OF TREATMENT OF HYPERTROPHIC SCARRING

a. Intralesional Steroids

In current clinical practice, corticosteroids injected locally into an area of hypertrophic scarring may produce flattening, decreased redness and softening of the scar. Presumably this occurs as a result of decreased collagen production and increased degradation as described above. The steroid usually used is triamcinalone (Kenalog) at a concentration of 40 mg/cc, injecting no more than 40 mg per session. This may be repeated every 4 to 6 weeks as necessary [2]. While this is useful for small localized lesions, systemic side effects of large doses of steroids limit their use in the widespread hypertrophic scarring seen in many burn patients.

b. Mechanical Pressure

Direct mechanical pressure as provided by various types of pressure garments inhibits development and enhances resolution of hypertrophic scarring [2,5]. The mechanism by which this occurs is not known but is hypothesized to be related to increased resorption of collagen relative to synthesis [37,38]. Pressure garments are worn continuously for 4-18 months until the wound lightens in colour and becomes flatter. Unfortunately, aggressive hypertrophic scarring often does not respond to this treatment. Furthermore, it is difficult to apply pressure to some regions of the body, for example orifices. Other drawbacks to this treatment modality are patient discomfort (garments may be hot, cause itching and limit mobility) and noncompliance (due to discomfort and feeling conspicuous).

c. Surgery

In situations where HTS causes limited mobility or narrowing of body orifices, surgery is sometimes indicated. This takes the form of excision of the scar and resurfacing or closure without tension of the resulting wound. As well, redirection of the axis of the scar by techniques such as Z-plasty may be helpful when joint mobility is reduced. Unfortunately, there is a tendency for hypertrophic scars to recur and thus enthusiasm for surgery should be balanced by the knowledge that the natural history of HTS is one of some degree of spontaneous resolution [2,3,5,6,7].

d. Topical Silicone Gel Sheets

Silicone gel sheets applied topically to areas of hypertrophic scarring seem to accelerate softening and resolution of the scars [39,40,41]. The mechanism of action of silicone is unclear. Quinn and associates [41,42] found that the beneficial effects of silicone are not due to mechanical pressure, change in temperature of the scar surface, change in oxygen tension in the wound or on its surface, or change in the degree of hydration of the wound surface. These investigators did note however, that when the gel sheet was placed on filter paper an oily print of the sheet was left on the paper. This material was examined with a scanning electron microscope X-ray analyzer and found to contain silicone. They thus wondered whether the mode of action of silicone gel was due to a chemical effect on the wound. Other investigators [39] have contested this idea with histologic studies of wounds treated with

silicone in which they could find no evidence of a foreign body reaction to suggest that silicone had entered the treated tissues.

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CHAPTER 4

POSSIBLE ROLE OF GAMMA INTERFERON IN THE CONTROL OF HYPERTROPHIC SCARRING

THE FAMILY OF INTERFERONS

The interferons are a family of proteins falling within the broad category of cytokines. Cytokines are low molecular weight protein cell regulators which provide short range communication between cells (usually in an autocrine or paracrine fashion rather than an endocrine fashion). Cytokines are produced by a wide variety of cells including monocytes/macrophages (specifically called monokines) and lymphocytes (specifically called lymphokines). Cytokines play an important role in communication between the cells involved in the inflammatory response and wound healing.

Interferons were originally described in the 1950's as proteins with antiviral activity, however since then they have been found to affect cell proliferation and modulate immune responses. There are several types of interferons: alpha, beta and gamma. Alpha interferon is produced by peripheral blood leukocytes or lymphoblastoid cells and inhibits viral replication and cell proliferation and modulates the immune response. Beta interferon is produced by fibroblasts in response to stimulation by live or inactivated virus. Gamma interferon is produced by antigen or mitogen stimulated T lymphocytes and has antiviral, antiproliferative and immunomodulatory activity. Natural gamma interferon is composed of 143 amino acids and has a molecular

weight of 16,000 to 25,000 daltons depending on the degree of glycosylation [1].

Clinically, gamma interferon has been used on an experimental basis as an antiviral agent and as an antineoplastic agent. In light of the fact that infiltrates of lymphocytes and monocytes are present in wounds undergoing both normal and pathological fibrosis, several investigators have looked at the effect of the monokine gamma interferon on fibroblast growth kinetics and collagen production. This will be discussed below.

THE EFFECT OF GAMMA INTERFERON ON GROWTH KINETICS AND COLLAGEN PRODUCTION IN NORMAL FIBROBLASTS

Pfeffer and colleagues [2] looked initially at the effect of interferon on cell growth kinetics. They used normal human fibroblasts in culture and subjected these to fibroblast-derived interferon (beta interferon) in concentrations from 10 to 2560 units/mL added continuously to the medium beginning 1 day after plating. They observed inhibition of fibroblast proliferation with concentrations greater than 40 U/mL; inhibition was maximal at 640 U/mL. They also looked at the effect of adding interferon to the medium for 24 hours only and noted that there was continued reduction in cell proliferation even after removal of interferon from the medium. Thus it was concluded that interferon has a prolonged antiproliferative effect on fibroblasts after even a short exposure.

Several other investigators have looked at the effect of gamma interferon on collagen production by normal dermal fibroblasts grown to confluence in culture. Jimenez and colleagues [3] showed that concentrations of 100 and 1000 U/mL reduced [^{14}C]-proline incorporation by 27.4 and 45.6%, respectively. This effect was independent of any effect on cell proliferation.

Another group of investigators, Duncan and Berman [4], studied the effect of gamma and beta interferon on subconfluent fibroblasts from normal adult dermis and normal neonatal foreskin. Contrary to the above experiments, they observed no inhibition of cell proliferation after 24 hours incubation with either interferon type. Instead they found that incubation for at least 48 hours was required to significantly inhibit fibroblast proliferation (as measured by [^{3}H] thymidine incorporation into DNA). They also looked at the effect of gamma and beta interferon on collagen production as measured by incorporation of [^{3}H] proline into collagen. It was found that both interferon types, at concentrations of 1000 U/mL or greater inhibited fibroblast collagen production by at least 50%. They looked finally at the effect of interferon on total protein production as measured by incorporation of [^{3}H]-tryptophan into TCA-precipitable protein, since tryptophan is not present in collagen molecules. There was found to be no reduction in noncollagenous protein synthesis. Thus, inhibition of collagen production by interferon is specific and not due to a general effect on fibroblast protein synthesis.

Tsutsumi and colleagues [5] looked at the effect of gamma interferon on normal human fibroblasts in culture and rat interferon on cultured rat hepatocytes. Incubation with interferon for 24 hours did not have any effect on proliferation of either cell type, as measured by [^{3}H] thymidine incorporation into DNA. It should be noted however that the fibroblasts were at confluence at the time of addition of interferon. Likewise, the hepatocytes were in a monolayer when the cytokine was added. In a second part of the experiment, they looked at collagen production as measured by [^{3}H] proline incorporation into collagenase-sensitive and collagenase-resistant protein which represent collagenous and noncollagenous proteins, respectively. There was noted to be a selective inhibition of collagen synthesis by both cell types when interferon was added to the media for 24 hours, even in concentrations as low as 22 U/mL.

Other investigators [6] have attempted to determine the level at which inhibition of collagen synthesis occurs. Confluent human fibroblasts were incubated with gamma interferon at a concentration of 1000 U/mL for 24 hours. This resulted in a selective inhibition of collagen without inhibition of non-collagenous proteins. Dot blot hybridization with a procollagen cDNA probe showed that the inhibition of collagen was accompanied by decreased levels of collagen mRNA.

Czaja and colleagues [7] attempted to delineate the level of regulation responsible for changes in procollagen mRNA steady-state levels using a nuclear run on assay. They grew normal human dermal fibroblasts to confluence in culture, then exposed these to gamma interferon (500 U/mL) for 24 hours. Total protein synthesis was not

changed, however collagen synthesis was reduced by 60%. RNA was extracted from the cells and steady-state collagen mRNA levels were determined by Northern and dot blot hybridization studies. Type I and type III procollagen mRNA levels were reduced to 23% and 7% of controls, respectively. Nuclear run-on studies showed no difference in transcription, suggesting that gamma interferon-induced decreases in procollagen mRNA are mediated through a post-transcriptional effect (such as enhanced turnover of mRNA).

In an effort to look at the effect of gamma interferon on collagen synthesis in vivo, Granstein and colleagues [8] produced full-thickness skin necrosis in mice using an argon laser. All mice had an osmotic pump implanted either subcutaneously or intraperitoneally. Half the pumps contained murine gamma interferon released at 8700 U/hour over 14 days, while control pumps contained no interferon. Animals were sacrificed at 14 and 21 days post-wounding. Examination by light microscopy and quantitative image analysis electron microscopy revealed less collagen in healing scars of interferon-treated mice. The time required to obtain full wound closure was delayed by approximately 25% in the interferon-treated mice. In addition, histologically, there was seen to be reduced polymorphonuclear cell infiltrate at the injury site in the interferon-treated animals.

THE EFFECT OF GAMMA INTERFERON ON CELLS THAT OVERPRODUCE COLLAGEN

Scleroderma is a disease of unknown etiology characterized by excessive deposition of collagen in the skin and various internal organs [9]. Rosenbloom and colleagues [10] looked at cultured fibroblasts from scleroderma patients and compared these to age- and sex-matched controls. They observed firstly that collagen production (as measured by incorporation of [^{14}C]- proline into peptide-bound hydroxyproline) was increased in the cells from scleroderma patients. The scleroderma cells were then exposed to gamma interferon in various concentrations. Greater than 50% inhibition of collagen production was noted with as little as 50 U/ml and maximal inhibition was seen with 500 U/ml (up to 80% inhibition). Examination of type I and type III procollagen mRNA levels revealed that both were reduced by the addition of gamma interferon (500 U/ml). Thus, as in the case of normal fibroblasts, interferon seems to decrease collagen biosynthesis in scleroderma fibroblasts by reducing levels of procollagen mRNAs.

In a similar set of experiments [11], fibroblast cultures were initiated from biopsies of affected skin in 5 scleroderma patients and 9 normal controls. It was observed that growth kinetics of the scleroderma fibroblasts were similar to those of the normal fibroblasts however collagen production was increased on average by 69% in the scleroderma cells. Subconfluent cultures were then incubated with or without 1000 U/ml of alpha, beta, or gamma interferon for 96 hours. Following this, the rates of cell proliferation in both normal and scleroderma cells were reduced by 50-60%; likewise, collagen production was reduced in both cell lines by 50-60%. Of note, there were no

IN VIVO STUDIES OF THE EFFECT OF GAMMA INTERFERON ON STATES OF COLLAGEN OVERPRODUCTION

Although keloid and hypertrophic scarring do not occur in animals, some investigators have looked at other situations in animals in which there is collagen overproduction. Granstein and colleagues [13] looked at fibrous capsule formation around osmotic pumps implanted subcutaneously in mice. Some of the pumps were loaded with recombinant rat interferon to be released by the pump at a rate of 2000 U/hour for 14 days. The remaining pumps in control animals contained no interferon. After 14 days the capsules from both animals were examined. The capsules surrounding the pumps containing interferon were grossly thinner and smaller than control capsules. By electron microscopy, interferon-exposed capsules were seen to contain thinner collagen bundles. Furthermore, fibroblasts within the capsules did not show evidence of active collagen synthesis. Finally, there was a significantly lower concentration of hydroxyproline in the interferon-exposed capsules as compared to controls. These results would suggest that gamma interferon may reduce collagen overaccumulation in vivo.

Others have looked at the effect of gamma interferon on another animal model of fibrosis, murine schistosomiasis [14]. During infection, the Schistosoma eggs become entrapped within the liver sinusoids leading to granuloma formation and ultimately hepatic fibrosis with excessive collagen deposition. In the first part of this study, Schistosoma-infected mice were compared to noninfected mice. Eight weeks post-infection when the mice were sacrificed, the infected livers were found to have an increased collagen content and increased

procollagen mRNA levels. Nuclear run-on assays suggested that this was a result of increased transcription. In the second part of the study, Schistosoma-infected mice received gamma IFN (100,000 U daily) by intramuscular injection for 4 weeks; control animals received no interferon. The mice were then sacrificed. Histological examination of the livers revealed less collagen deposition in the livers of the interferon-treated animals. The interferon-treated mice livers also had a lower collagen content (as measured by hydroxyproline content) than controls. Northern blot analysis showed types I and III procollagen mRNA levels in interferon-treated mice livers to be reduced to approximately 30% of controls. Nuclear run-on assays suggested that these reduced mRNA levels were due to post-transcriptional regulation rather than altered transcription as was the case in the first part of the experiment where Schistosoma infection was observed to have increased the rate of transcription.

Castilla and colleagues [15] have observed in a small group of patients with chronic hepatitis and cirrhosis that treatment for one year with alpha interferon reduced hepatic fibrogenic activity.

There has also been an attempt in humans to use subcutaneous gamma interferon as an antiproliferative agent in rheumatoid arthritis to decrease collagen production by synovial cells [16]. This was a double-blind study in which 22 patients received subcutaneous gamma interferon or placebo. The patients treated with interferon received 100 micrograms daily for the first week, then 100 micrograms 2 times per week for 23 further weeks. No serious adverse reactions were noted with this regimen. When other variables were controlled, the patients

treated with interferon were noted to have significantly less joint tenderness and fewer subcutaneous nodules than untreated patients. Although this was a small study looking at qualitative parameters, it demonstrates the clinical use of gamma interferon for an inflammatory disorder without serious side-effects.

In another study, Berman and colleagues [17] studied a single enlarging keloid on the arm of 55 year old male which developed 10 weeks after a carbon dioxide laser test dose. A biopsy was first obtained from the lesion and cultured fibroblasts grown from this. The lesion itself was then treated with 1.5 million units of alpha interferon injected into the lesion. This treatment was repeated 4 days later. Nine days after the initial injection another biopsy for cell culture was taken. A biopsy was also taken from a normal area of the patient's skin. Pretreatment keloid fibroblasts demonstrated normal growth kinetics but produced more collagen than normal cells. In contrast, post-treatment keloid fibroblasts demonstrated collagen production rates similar to normal fibroblasts. It was noted that clinically the keloid lesion decreased in area by approximately 40%. Again it was observed that the patient experienced no side-effects from this treatment except for transient myalgias which resolved without specific treatment.

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CHAPTER 5

THE EFFECT IN VITRO OF GAMMA INTERFERON ON CELL PROLIFERATION, COLLAGEN PRODUCTION AND PROCOLLAGEN mRNA EXPRESSION IN HYPERTROPHIC SCAR FIBROBLASTS

INTRODUCTION

Hypertrophic scarring is an abnormal form of wound healing characterized by overabundance of tightly-packed collagen within the extracellular matrix [1,2,3,4]. Clinically this is seen as a red, raised, nodular, inelastic scar which in contrast to normal scars undergoes a prolonged and incomplete regression [5]. Hypertrophic scarring may occur following almost any cutaneous injury extending into the dermis but is particularly problematic in burn injured patients, who often have extensive areas of healing wounds. In addition to being cosmetically unacceptable, hypertrophic scarring may become a functional problem by limiting mobility of joints and areas of normally loose skin or by narrowing body orifices [6].

The pathophysiology of this disorder is incompletely understood however appears to be related to an imbalance between collagen production by fibroblasts and collagen degradation [7]. Since hypertrophic scarring is a process unique to humans, a good animal model does not exist [8] and therefore most research in this area has involved cultured fibroblasts. Some investigators have observed increased collagen production by cultured hypertrophic scar fibroblasts as compared to fibroblasts from the skin of normal subjects [9,10]. On the contrary, Abergal and colleagues [11] found that only 4 of 9

hypertrophic scar cell strains where overproducers of collagen and still others [12,13] have found there to be no increase in collagen production by hypertrophic scar fibroblasts.

In vivo, the inflammatory response constitutes an early phase in the wound healing process and it is thus believed that various monokines and lymphokines play a role in the initiation, evolution and resolution of scarring [14,15]. In support of this notion is experimental evidence showing that interleukin-1 and transforming growth factors alpha and beta increase collagen production in cultured fibroblasts [16,17,18]. Conversely, gamma interferon, a lymphokine produced by activated T-lymphocytes is known to inhibit fibroblast proliferation and reduce fibroblast collagen production by cultured fibroblasts [18]. Clarification of the roles of these cytokines might improve the understanding of hypertrophic scarring as well as other states of fibrosis such as pulmonary fibrosis, hepatic cirrhosis and scleroderma.

Presently, problematic hypertrophic scars are treated with mechanical pressure [19,20,21,22], silicone gel sheets [23,24,25] and, less commonly, intralesional corticosteroid injections [19]. In situations of extensive hypertrophic scarring with limited joint mobility or narrowed body orifices, surgery is sometimes contemplated. This takes the form of scar excision with wound resurfacing. Unfortunately there is a tendency for hypertrophic scars to recur following surgery such that one's enthusiasm for this approach should be tempered [4,8,19,20,26].

There has been recent interest in the potential use of gamma interferon or other interferons for pharmacologic control of various states of fibrosis. Gamma interferon has been shown to inhibit cell proliferation and collagen production in cultured normal fibroblasts [15,18,27,28]. Other investigators, again in cultured normal fibroblasts, have shown reduced procollagen mRNA levels in response to gamma interferon [29]. In mice, gamma interferon decreases collagen synthesis around a subcutaneously implanted foreign body [30] and in Schistosoma-induced hepatic fibrosis [31]. In addition it has resulted in clinical improvement in several small human studies of keloid scarring [32,33], scleroderma [34] and hepatic cirrhosis [35].

The present study looks at the effect <u>in vitro</u> of gamma interferon on fibroblast cell strains derived from human hypertrophic burn scars and unaffected dermis from the same patients. Specifically, the influence of gamma interferon upon cell proliferation, collagen production and procollagen mRNA levels is examined.

MATERIALS AND METHODS

a. Source of Gamma Interferon

A preparation of human recombinant gamma interferon (antiviral activity: 1.3×10^7 IU/mL determined by WISH/VSV cpe microtiter assay standardized with NIH HuIFN-gamma reference standard number Gg 23-901-530) was kindly supplied by Dr. Michael Brunda (Hoffman-LaRoche Inc., NJ)

b. Clinical Specimens

Specimens for study were obtained from five patients seen at the University of Alberta Hospitals with hypertrophic scarring developing subsequent to a cutaneous thermal injury (Table 1). All patients demonstrated extensive areas of hypertrophic scarring as manifested by raised, erythematous, pruritic, thickened and noncompliant scars confined to the site of injury. In some cases scarring resulted in functional impairment in the form of restricted range of motion. The patients, all males, ranged in age from four to forty-nine years and sustained their burn injuries from four to eight months prior to the time of sampling. After informed consent, small biopsies were obtained from an area of hypertrophic scarring either surgically or using a 5 mm biopsy punch. In addition, a site-matched control biopsy was obtained from uninjured skin in a corresponding anatomic location in each of the patients (Fig. 1).

c. Cell Cultures

Fibroblast cultures were prepared from biopsies by the explant method as described by Nakano and Scott [36]. Briefly, biopsies were collected individually and washed three times in sterile Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) supplemented with antibiotic-antimycotic preparation (100 U/mL penicillin, 100ug/mL streptomycin, 0.25ug/mL amphotericin B) (Gibco). Specimens were then dissected free of fat and minced into small pieces less than 0.5 mm in diameter, washed six times with DMEM, and distributed onto 60 x 15 mm petri dishes, four pieces per dish. A sterile glass coverslip was

placed over the tissue fragments and fixed to the dish with a small amount of sterile silicone grease in order to immobilize the fragments. Four milliliters of DMEM supplemented with antibiotic-antimycotic and 10% fetal bovine serum (FBS) (Gibco) (DMEM-10% FBS) was added to each dish and these were incubated in an atmosphere of 5% CO_2 in air at 95% relative humidity and 37°C. Medium was replaced weekly for four weeks by which time fibroblast outgrowth had covered more than 50% of the growth surface. At this time the fibroblasts were released from the dishes by brief (less than 5 minutes) treatment with 0.25% trypsin and subsequently seeded into Corning 75 cm^2 culture flasks (Corning, NY) in DMEM-10% FBS, then incubated. Upon reaching confluence, the cells were released by trypsinization, split for subculture at a ratio of 1:6 and reseeded into 75 cm^2 flasks. Cells at passage 3 or 4 were used to look at the effect of gamma interferon on fibroblast proliferation, hydroxyproline production and procollagen mRNA levels.

d. Evaluation of Fibroblast Growth Kinetics

Normal and hypertrophic scar fibroblasts were seeded onto Corning 24-well cell culture plates (Corning, NY) in DMEM-10% FBS at an initial cell density of 3.5×10^3/mL. In a pilot experiment, cells from one cell strain (strain 2) were incubated for 48 hours after which the medium was replaced with fresh medium (1 mL/well) containing varying concentrations of gamma interferon (0 U/mL, 500 U/mL, 1000 U/mL, 2000 U/mL, 4000 U/mL). On days 1, 2, 4, 7, 11, 15 and 20 post-plating, cells were released from some wells by brief treatment with 0.25% trypsin and a cell count was determined using a Coulter counter (Coulter

Electronics, Hialeah, FL). Based on information obtained from this cell strain, experiments on the subsequent four cell strains involved addition of gamma interferon after 48 hours at doses of 0 U/mL, 1000 U/mL and 2000 U/mL with cell counts obtained on days 7 and 11 post-plating. There were four replicates for each day and experimental condition.

e. Evaluation of Fibroblast Collagen Production

Normal and hypertrophic scar fibroblasts were seeded onto 6-well tissue culture plates (Falcon, Lincoln Park, NJ) in 2.0 mL DMEM-10% FBS at an initial cell density of 3.0×10^4 cells/mL. The medium was changed every five days. When both hypertrophic and normal cells had reached visual confluence (day 9-12), the medium was removed and the cell monolayer was rinsed with 2.0 mL sterile phosphate buffered saline (PBS). Two mL of sterile "test medium" were then added to each well. The test medium consisted of: DMEM, 10% dialyzed FBS (dialyzed four times against 0.9% sodium chloride using Spectropor membrane tubing (Spectrum Medical Industries, Los Angeles, CA) with a molecular weight cutoff of 12-14,000), 0.1 mM proline, 50 ug/mL ascorbic acid, 50 ug/mL beta-aminopropionitrile HCl [37]. In addition, in half of the wells the test medium included gamma interferon (1000 U/mL) while in the remaining wells no interferon was added. There were four replicates for each experimental condition. In addition test medium with and without interferon was added to four blank wells. The dose of gamma IFN used was based upon previous studies in which 1000 U/mL resulted in consistent reduction of collagen production by normal fibroblasts

[15,28,29] and a pilot experiment in which HTS fibroblasts from one cell strain (strain 2) were grown in 24-well plates and treated with test medium (as described above) including 0, 1000, 2000 or 4000 U/mL gamma IFN. The cells and blank wells were incubated for 48 hours in an atmosphere of 5% CO_2 in air at 95% relative humidity and 37°C. After this time the medium was harvested from each of the wells and stored at -20°C until later analysis for collagen content by gas chromatography/mass spectrometry. Only the culture medium was analyzed since, as shown previously, more than 85% of the total collagen produced by cultured fibroblasts is released into the medium under the conditions used [38]. The cells were collected from the wells by trypsinization and counted using the Coulter counter.

f. Collagen Analysis by Gas Chromatography/Mass Spectrometry

Fibroblast collagen production was evaluated by quantitation of the amount of hydroxyproline present in the medium by gas chromatography/mass spectrometry as described previously by Tredget et al. [39]. Briefly, 10 ul of 25 ng/ul 5-hydroxy-L-pipecolic acid (an internal standard) was added to each sample. The samples were in turn dried in a centrifugal evaporator (Speed-Vac, Savant Instruments Inc., Farmington, NY) with a refrigerated condensation trap. The samples were then hydrolysed with 0.5 mL 6M HCl at 116°C for 16 hours. The hydrolysate was then dried, again in the centrifugal evaporator. The samples were next esterified with Methanol:HCl (4:1 molar ratio, made fresh daily) at 80°C for 30 minutes, thus creating a methyl ester derivative of each amino acid. Trifluoroacetic anhydride was then added

and the samples heated at 60°C for 10 minutes followed by cooling to room temperature, drying and redissolution in ethyl acetate. The final derivative of hydroxyproline thus obtained was the N,O-trifluoroacetyl N-methyl ester and of hydroxypipecolic acid the N-trifluoroacetyl N-methyl ester. Gas chromatography/mass spectrometry was performed using a Hewlett-Packard 5890A Gas Chromatograph linked to a Hewlett-Packard 5970 Mass Selective Detector. This device was used to monitor the ions m/z 164 and 278 which are characteristic of N,O-trifluoroacetyl N-methyl hydroxyproline and the ion m/z 179 which is characteristic of the internal standard N-trifluoroacetyl N-methyl hydroxypipecolic acid. Each sample was run in duplicate. By reference to a standard curve of the internal standard (hydroxypipecolic acid) analyzed under similar conditions, it was thus possible to determine the amount of hydroxyproline present, expressed as pg hydroxyproline/cell/48 hours.

g. RNA Extraction and Hybridization

Normal and hypertrophic scar fibroblasts were seeded into Corning 75 cm^2 tissue culture flasks (Corning, NY) in DMEM-10% FBS and incubated in 5% CO_2, 95% relative humidity and 37°C. Medium was changed weekly. When both normal and hypertrophic scar fibroblasts had reached visual confluence, the culture medium was removed and replaced with 10 mL "test medium" consisting of DMEM, 10% dialyzed FBS, 0.1 mM proline, 50 ug/mL ascorbic acid, 50 ug/mL beta-aminopropionitrile and variable concentrations of gamma interferon. The initial set of experiments involved the addition of gamma interferon in doses of 0, 1000 and 2000 U/mL for 6, 12 and 24 hours. Results from these experiments

demonstrated maximal effect with 1000 U/mL for a treatment period of 12 hours. Other investigators have demonstrated, in normal fibroblasts, a consistent reduction in procollagen mRNA level with 1000 U/mL gamma IFN [29]. Subsequent experiments thus involved the addition of either 0 or 1000 U/mL of gamma interferon to the test medium. After incubation for 12 hours the medium was removed and the cell layers were washed twice with 10 mL of ice-cold PBS. Fibroblasts from 6 flasks were lysed with and pooled in 6 mL 4 M guanidinium thiocyanate (GITC). The cell lysate was transferred to a 50 mL Corning plastic tube (Corning, NY) where cellular DNA was sheared using a 10 mL syringe with 18 gauge needle (40 strokes). Total RNA was extracted from the lysates (in GITC) by layering onto a cushion of 3 M CsCl followed by centrifugation in a TI 50 rotor (Beckman Industries Inc., Palo Alta, CA) at 25,000 rpm for 18 hours [40]. The resulting total RNA for each experimental condition was separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde. The RNA was then blotted from the gel onto a nitrocellulose filter by capillary transfer using 20x SSC (1x SSC = 0.15 M NaCl, 0.015 M Na citrate) [41] and baked for 2 hours at 80^{o}C in a vacuum oven. The filter was treated for 4-6 hours at 42^{o}C with a pre-hybridization solution containing 50% formamide, 20 mM NaH_2PO4 (pH 7.0), 4x SSC, 2 mM EDTA, 4x Denhardt's solution (1x = 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 1% SDS and 100 ug/mL sonicated denatured salmon sperm DNA. Hybridization was performed at 42^{o}C for 16-20 hours in the same solution to which had been added either pro alpha 2 (I) or pro alpha 1 (III) cDNA probes (kindly provided by Drs. G. Tromp, H. Kuivaniemi and L. Ala-Kokka, Department of Biochemistry and Molecular

Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA). In addition, in most cases a cDNA probe for the heat shock-like protein, Nb29, [42] was also added to the hybridization solution as a loading control. In other cases the gels were stained with ethidium bromide and photographed under ultraviolet light to serve as a loading control. In some cases both ethidium bromide staining of the gel and probing of the corresponding blot with Nb29 cDNA were carried out to ensure that Nb29 expression was not affected by gamma IFN, confirming its usefulness as a loading control. The cDNA probes were labelled with ^{32}P-alpha-dCTP by the nick-translation technique. The filters were subsequently washed for one hour at room temperature with 2x SSC and 0.1% SDS and then with 0.1x SSC and 0.1% SDS for 30 minutes at 65^{o}C. Autoradiography was performed by exposing Kodak X-Omat film (Kodak, Edmonton, Alberta) to the nitrocellulose filters at -70^{o}C. The autoradiograms were then analyzed by quantitative densitometry.

h. Statistical Methods

Comparisons between treated and untreated cells and between hypertrophic scar cells and their paired normal controls were made using two-way analysis of variance (ANOVA) on repeated measures, thus taking into account pairing of normal and HTS cells. A p value of <0.05 was considered to be statistically significant. All results are expressed as the mean $\pm$ standard error of the mean (SEM).

RESULTS

a. Effect of Gamma Interferon on Cell Proliferation

The effect of gamma IFN on fibroblast proliferation was initially studied in one cell strain (strain 2) in a pilot experiment involving varying concentrations of gamma IFN (0, 500, 1000, 2000 or 4000 U/mL) added 48 hours after plating of the cells. Serial cell counts demonstrated an inhibition of proliferation in both normal and HTS cells (Fig. 2). This effect was marked with 1000 U/mL gamma IFN and became slightly more pronounced with higher concentrations.

Subsequent experiments looking at all 5 cell strains involved the addition of gamma IFN (0, 1000 or 2000 U/mL) again 48 hours after cell plating with cell counts obtained on days 7 and 11 (5 and 9 days after addition of IFN, respectively) (Fig. 3). Of initial interest, there was no statistical difference in rates of proliferation between untreated normal fibroblasts and untreated HTS fibroblasts. When treated with 1000 U/mL gamma IFN, there was found to be a statistically significant inhibition of both normal and hypertrophic scar fibroblast proliferation by day 7 (65 and 49% of untreated state, respectively) which was still evident by day 11 (68 and 50% of untreated state, respectively) (Fig. 3). Treatment with 2000 U/mL gamma IFN did not cause significant further inhibition. There was no statistical difference in the degree of inhibition between hypertrophic scar cells and their normal controls. Thus, gamma IFN seems to inhibit proliferation of HTS and normal fibroblasts to the same extent. Raw data for these experiments on cell proliferation are shown in Appendix 1 and Appendix 2.

b. Effect of Gamma Interferon on Fibroblast Collagen Production

A pilot experiment was conducted in which confluent HTS fibroblasts were treated with varying doses of gamma IFN (0, 1000, 2000 or 4000 U/mL). After 48 hours incubation the medium was examined for hydroxyproline content (Fig. 4). There was a pronounced reduction in hydroxyproline production by those cells treated with 1000 U/mL IFN. Higher doses however did not cause a further reduction. Other investigators have also shown consistent reduction in collagen production by normal and scleroderma fibroblasts with gamma IFN at a concentration of 1000 U/mL [15,28,29,43,44]. This concentration was thus selected for the following set of experiments involving all 5 cell strains.

The hydroxyproline content of the media (representative of collagen) from confluent normal and hypertrophic scar fibroblasts after 48 hours incubation with 1000 U/mL gamma IFN is shown in Figure 5. Both normal and hypertrophic scar cells show statistically significant reductions in hydroxyproline production with the addition of 1000 U/mL gamma IFN (75 and 66% of untreated controls, respectively). The degree of this reduction was not however significantly different between normal and HTS cells. Of additional note, while the mean collagen production for untreated HTS cells was 3.7 pg/cell/48 hours versus 5.7 pg/cell/48 hours for untreated normal controls, this difference was not statistically significant due to the high variability between cell strains. Raw data for these experiments on collagen production are shown in Appendix 3.

c. Effect of Gamma Interferon on Expression of Type I and Type III Procollagen mRNA Levels

Two preliminary studies were done to determine suitable treatment conditions for this set of experiments looking at the effect of gamma IFN on mRNA levels. Firstly, confluent cells from 2 cell strains (strains 2 and 3) were incubated with test medium with and without 1000 U/mL gamma IFN for varying time periods (6, 12 and 24 hours). Figure 6 (panel A) shows the autoradiogram from a Northern analysis of pooled total RNA from the 2 cell strains probed with cDNA specific for mRNA coding for the pro alpha 2 (I) chain of type I collagen. The probe is seen to visualize 2 transcripts (5.8 kb and 4.8 kb) similar to those previously described for type I procollagen [45]. Ethidium bromide staining of 28S and 18S ribosomal RNA was used as a loading control (Fig. 6B). With gamma IFN, there is clear reduction in message level at 12 hours in both normal and HTS cells. Test medium alone did not affect mRNA level (Fig. 6C and 6D).

In a second preliminary study cells from one cell strain (strain 2) were treated for 12 hours with gamma IFN, either 1000 U/mL or 2000 U/mL, and procollagen mRNA levels were compared to those from untreated cells. Figure 7 (panel A) shows the autoradiogram from the Northern analysis of total RNA probed with the cDNA for the alpha 2 (I) chain of type I collagen, again visualizing 2 transcripts (5.8 kb and 4.8 kb). A cDNA probe for the heat-shock-like protein, Nb29, was used as a loading control and can be seen as a 2.4 kb transcript. After quantitative densitometry and correction for the ratio of pro alpha 2 (I) to Nb29 mRNA, it can be seen (Fig. 7B) that gamma IFN at a concentration of 1000

U/mL causes a marked reduction in type I procollagen mRNA in both normal and HTS cells (31% and 34% of untreated state, respectively). Treatment with 2000 U/mL caused only a slight further reduction in mRNA levels. Figure 8 (panel A) shows the autoradiogram from the Northern analysis of total RNA probed with cDNA specific for mRNA coding for the alpha 1 (III) chain of type III collagen, in this case visualizing 2 transcripts (5.4 kb and 4.8 kb). Nb29 cDNA was again used as a loading control and can be seen as a 2.4 kb transcript. It can be seen (Fig. 8B) that gamma IFN at a concentration of 1000 U/mL causes a marked reduction in type III procollagen mRNA in both normal and HTS cells (46% and 65% of untreated state, respectively). Treatment with 2000 U/mL does not further reduce mRNA levels in the normal cells and has only a slightly more pronounced effect (statistically insignificant) on the HTS cells. Other investigators have also shown consistent reduction in procollagen mRNA levels in normal fibroblasts with 1000 U/mL [29].

Figure 9 (panel A) is an autoradiogram of the Northern blot of gamma IFN treated and untreated cells from strain 5, hybridized with the cDNA probe for the alpha 2 (I) chain of type I procollagen and with the cDNA probe specific for Nb29. Panel B demonstrates ethidium bromide staining of 28S and 18S ribosomal RNA when the gel was placed under ultraviolet light prior to its transfer to nitrocellulose and is added to demonstrate equal loading of the samples. It can be seen that although gamma IFN (1000 U/mL) reduces type I procollagen mRNA in both normal and HTS cells, it does not affect expression of Nb29 message, thus confirming the usefulness of the latter as a loading control in these experiments.

Based on the above findings, the remaining 4 cell strains were treated with 1000 U/mL gamma IFN for 12 hours. The relative abundance of type I procollagen mRNA in treated and untreated cells was determined individually for each cell strain by Northern blot analysis. The results of these analyses after quantitative densitometry and correction for loading are shown in figure 10 (panel B). Panel A is the autoradiogram from a Northern analysis of pooled total RNA from the 5 cell strains (2.5 ug from each cell strain under each treatment condition). Again, the cDNA probe is specific for mRNA coding for the pro alpha 2 (I) chain of type I collagen and visualizes two transcripts (5.8 kb and 4.8 kb) while that for Nb29 (loading control) can be seen as a 2.4 kb transcript. Of initial note, there was not a significant difference in message levels between untreated normal and hypertrophic scar fibroblasts. Treatment with gamma IFN (1000 U/mL for 12 hours) however, significantly reduced the level of type I procollagen mRNA in both normal and hypertrophic scar fibroblasts (50 and 45% of untreated state, respectively). The degree of this reduction was not, however, significantly different between the normal and hypertrophic cells. Raw data for these experiments looking at type I procollagen mRNA are shown in Appendix 4.

The relative abundance of type III procollagen mRNA in gamma IFN treated and untreated normal and hypertrophic scar cells was again determined individually for each of the 5 cell strains. These results after quantitative densitometry and correction for loading are shown (Fig. 11B) again with an autoradiogram of the Northern analysis of pooled RNA (2.5 ug from each of the 5 cell strains under each treatment

condition) (Fig. 11A). Here, the cDNA probe is specific for pro alpha 1 (III) mRNA and visualizes 2 transcripts (5.4 kb and 4.8 kb). Again, a cDNA probe specific for Nb29 was used as a loading control and is seen as a 2.4 kb transcript. There was not a statistically significant difference in message level between untreated normal and HTS cells, however exposure to gamma IFN (1000 U/mL for 12 hours) caused significant reduction in type III procollagen mRNA in both normal and hypertrophic scar fibroblasts (62 and 64% of untreated state, respectively). The degree of reduction was again not significantly different between normal and hypertrophic scar cell types. Raw data for these experiments looking at type III procollagen mRNA are shown in Appendix 5.

DISCUSSION

While hypertrophic scarring may develop consequent to any traumatic or surgical injury, it is particularly problematic following thermal injury. Improvements in the early management of burn patients have significantly reduced mortality from this condition. A natural consequence has been a larger number of patients surviving extensive burns and entering the rehabilitative phase of treatment. Hypertrophic scarring poses a significant cosmetic and functional problem for such people. At the tissue level, it is a condition of overaccumulation of collagen within the dermis of the skin and as such is not unlike fibrotic conditions elsewhere in the body such as scleroderma, hepatic fibrosis, pulmonary fibrosis and keloid [1].

Current treatment modalities for this problem include mechanical pressure in the form of pressure garments and topical application of silicone gel sheets [4,46]. While these are relatively innocuous treatments they are often inadequate for problematic scarring, even when used diligently. Intralesional corticosteroid injections may improve isolated lesions however they are also associated with dermal thinning and epidermal atrophy and are not practical for widespread hypertrophic scarring [47]. Surgical excision and resurfacing of the lesions is frequently complicated by recurrence [4].

As mentioned earlier, various cytokines are felt to play a role in regulation of collagen metabolism within healing wounds. The lymphokine gamma interferon, which has been shown to reduce fibroblast collagen production seems attractive as a potential agent for control of hypertrophic scarring.

This series of experiments studied the <u>in vitro</u> effect of gamma IFN on 5 hypertrophic scar cell strains using normal dermal fibroblasts obtained from the same patients as controls. Treatment with gamma IFN (1000 U/mL) resulted in significant inhibition of cell proliferation in both normal (to 65% of untreated state after 5 days) and hypertrophic scar fibroblasts (49% of untreated state after 5 days), with the degree of inhibition being the same for HTS cells and normal cells. Of greater interest, treatment with gamma IFN (1000 U/mL) resulted in reduced collagen production in both normal (75% of untreated state) and HTS fibroblasts (66% of untreated state). HTS cells were not differently than normal cells. It would appear that reduced collagen production is at least in part due to reduced procollagen mRNA levels. Treatment with

gamma IFN (1000 U/mL) was associated in both normal and HTS cells with reduced type I (50 and 45%, respectively) and to a lesser degree type III (62 and 64%, respectively) procollagen mRNA levels. Again there was not a significant difference in the degree of this reduction between the hypertrophic scar fibroblasts and their normal controls.

It would thus appear that, at least in vitro, gamma IFN exhibits an inhibitory effect on cell proliferation and collagen production and reduces procollagen mRNA levels in hypertrophic scar fibroblasts. This effect is at least as great in the HTS cells as it is the normal fibroblasts. If this is also the case in vivo, there is potential for therapeutic use of gamma IFN in the control of problematic hypertrophic scarring.

There have in fact been clinical studies in which a small number of patients with hypertrophic or keloid scars were treated with intralesional gamma IFN with clinical improvement of the condition [33,47]. There have also been clinical studies in which gamma IFN has been safely administered systemically to small groups of patients with scleroderma [34] and rheumatoid arthritis [48] with clinical improvement in their disease.

These experiments demonstrate that in the cell culture environment, HTS fibroblasts behave much the same as their normal controls not only in terms of rate of proliferation, collagen production and procollagen mRNA levels in the untreated state, but also in their response to the cytokine gamma IFN. While this in vitro model is useful in studying the isolated effect of gamma IFN on the different cell types, it may not accurately reflect the in vivo environment. It seems

possible, from these studies, that HTS fibroblasts do not differ (at least in terms of the parameters studied) from normal dermal fibroblasts but rather are responding to local stimuli particular to the milieu of the healing wound. On the other hand these findings may be artifacts of the *in vitro* state; that is the culture environment may be selecting for a particular cell strain that behaves in this fashion.

In summary, in an *in vitro* environment gamma IFN significantly reduces cell proliferation, collagen production and procollagen mRNA levels in HTS fibroblasts to the same degree as fibroblasts from normal dermis. This supports the idea of administering gamma IFN to patients for the control of problematic hypertrophic scarring. Future studies in this area might be directed at understanding the effect of the actual wound milieu on fibroblasts and looking at the effect of systemically administered gamma IFN on both normal fibroblasts and those within the healing wounds.

Table 1: Clinical Characteristics of Patients Used as Sources For Paired Cell Lines

Patient (Cell Strain)	Sex	Age at Time of Burn Injury	% of Total Body Surface Area Burned	Time Interval Between Burn Injury and Biopsy
1	Male	18 Years	75	8 months
2	Male	49 Years	28	4 Months
3	Male	31 Years	60	5 Months
4	Male	4 Years	25	5 Months
5	Male	12 Years	35	7 Months

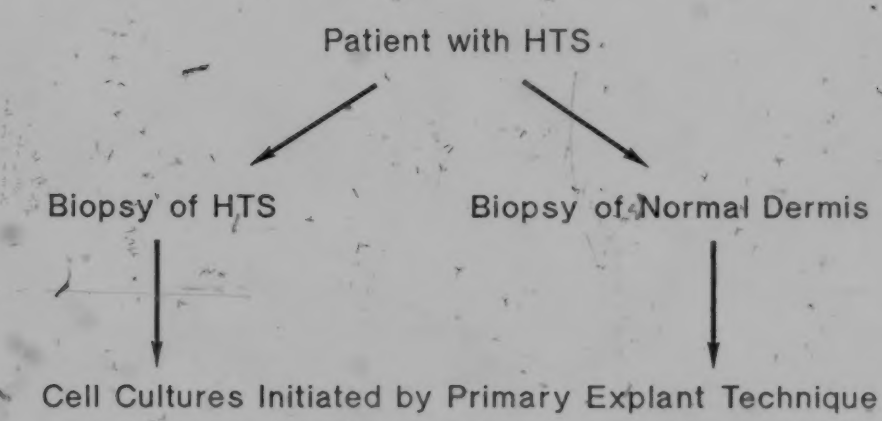

Figure 1. Initiation of paired cell strains from patients with hypertrophic scarring. From each patient simultaneous biopsies were obtained from an area of hypertrophic scarring and an area of unaffected dermis. Cell cultures were subsequently initiated from these biopsies.

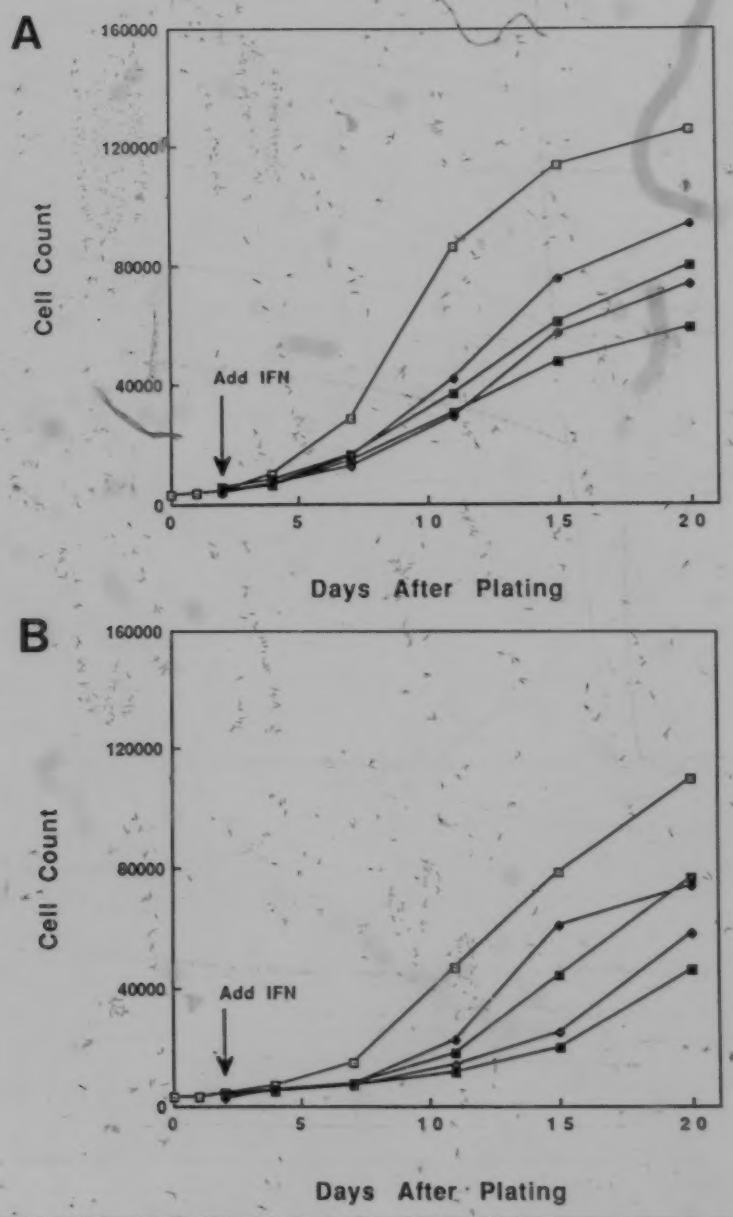

Figure 2. Effect of different doses of gamma interferon on growth kinetics of normal (panel A) and hypertrophic scar (panel B) fibroblasts from cell line 2. Depicted are serial cell counts after treatment with varying concentrations of gamma IFN: 0 U/ml (⊡), 500 U/ml (♦), 1000 U/ml (◘), 2000 U/ml (◊), 4000 U/ml (■).

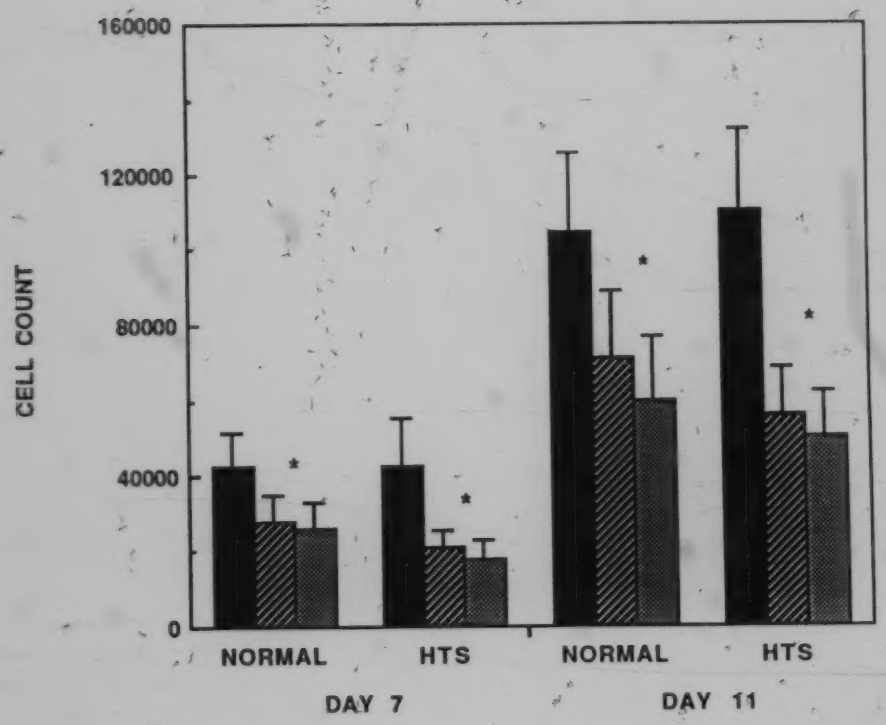

Figure 3. Effect of gamma interferon on fibroblast proliferation amongst 5 paired normal/ hypertrophic cell lines. Cells were treated on day 2 post-plating with: no gamma IFN (■), 1000 U/ml gamma IFN (▨) or 2000 U/ml gamma IFN (■); cell counts were obtained on days 7 and 11. Data represent means ± SEM for 5 cell pairs. Cells treated with gamma IFN were compared to their respective untreated controls (* $p<0.05$)

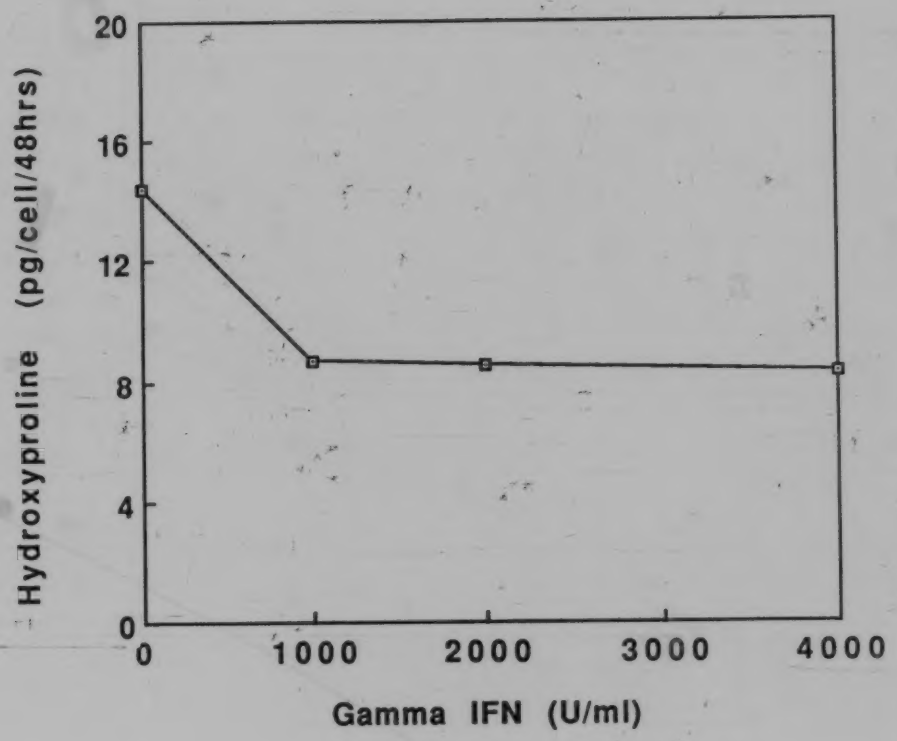

Figure 4. Dose effect of gamma interferon on collagen production by hypertrophic scar fibroblasts from cell line 2. Collagen production is assessed by determination of hydroxyproline production by confluent cells over a 48 hour period.

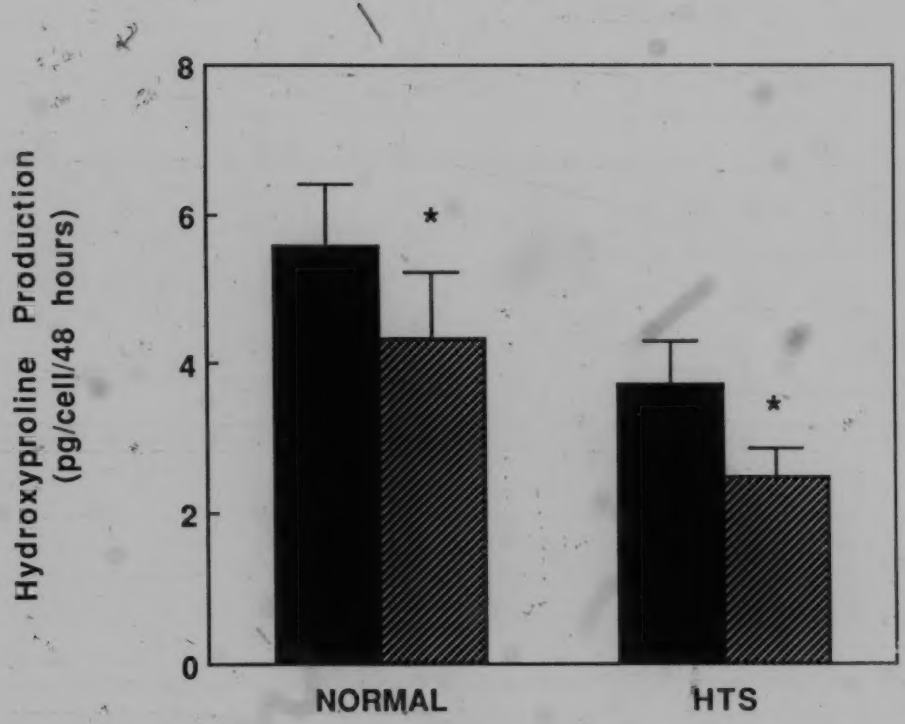

Figure 5. Effect of gamma interferon on collagen production by 5 paired normal/hypertrophic scar cell lines. Cells were treated with either no interferon (■) or 1000 U/ml (▩). Determination of collagen production is based upon hydroxyproline production by confluent cells over a 48 hour period. Data represent mean ± SEM for 5 pairs of cells. Treated cells were compared to their untreated controls (* $p<0.05$).

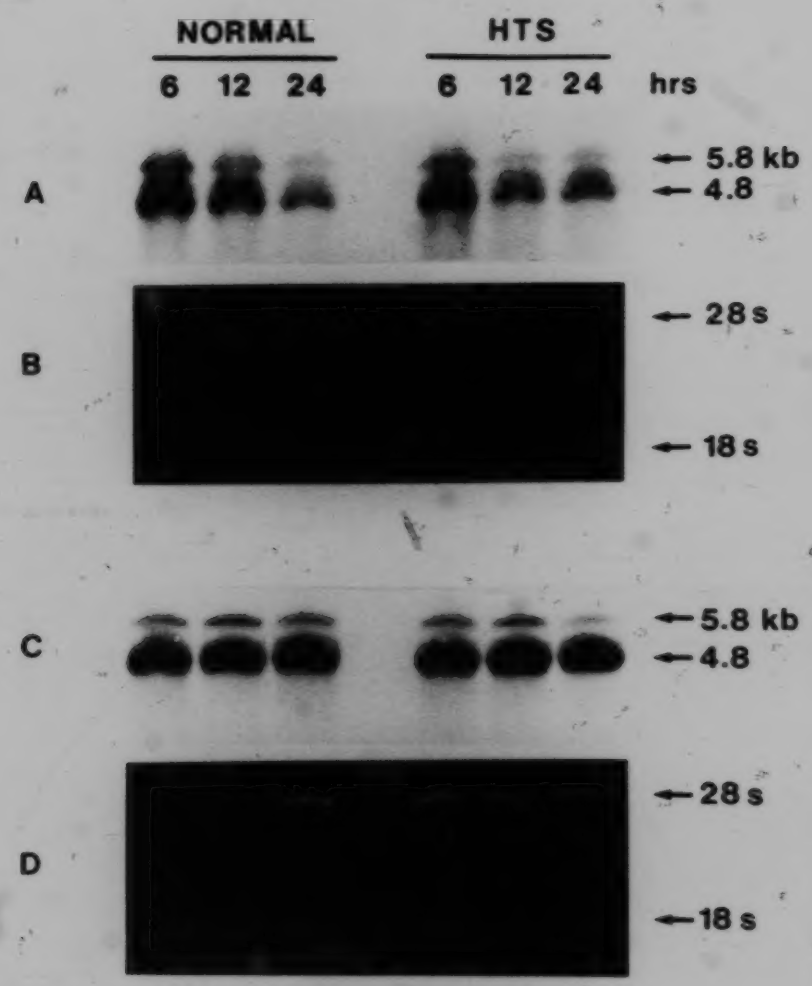

Figure 6. Effect of gamma interferon treatment duration on type I procollagen mRNA expression in normal and hypertrophic scar fibroblasts from cell strain 2. Total RNA of fibroblast cultures was extracted and electrophoresed on a 1% agarose gel (7.5 ug/lane) then hybridized with pro alpha 2 (I) cDNA. Panel A demonstrates the effect of test medium plus IFN (1000 U/ml) for various time periods along with its ethidium bromide stained gel (panel B) as a loading control. The effect of treatment with test medium alone for various time periods is shown in panel C along with its corresponding ethidium bromide stained gel for loading control (panel D).

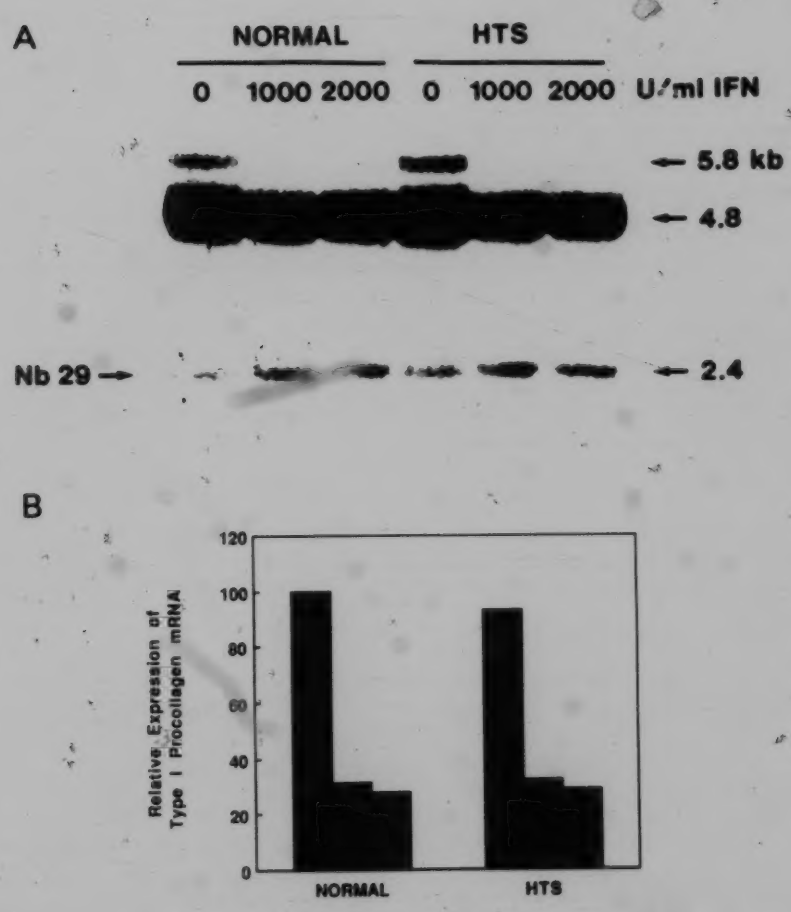

Figure 7. Effect of gamma interferon on type I procollagen mRNA expression in normal and hypertrophic scar fibroblasts from cell strain 2. Panel A shows the autoradiogram of the Northern blot of RNA from cells treated for 12 hours with varying doses of gamma IFN. Nb29 mRNA expression is included as a loading control. Relative expression of type I procollagen mRNA can be determined by quantitative densitometry after correcting for variation in loading (panel B); bars on the histogram represent cells receiving no treatment (■), 1000 U/mL gamma IFN (▨) or 2000 U/mL gamma IFN (■).

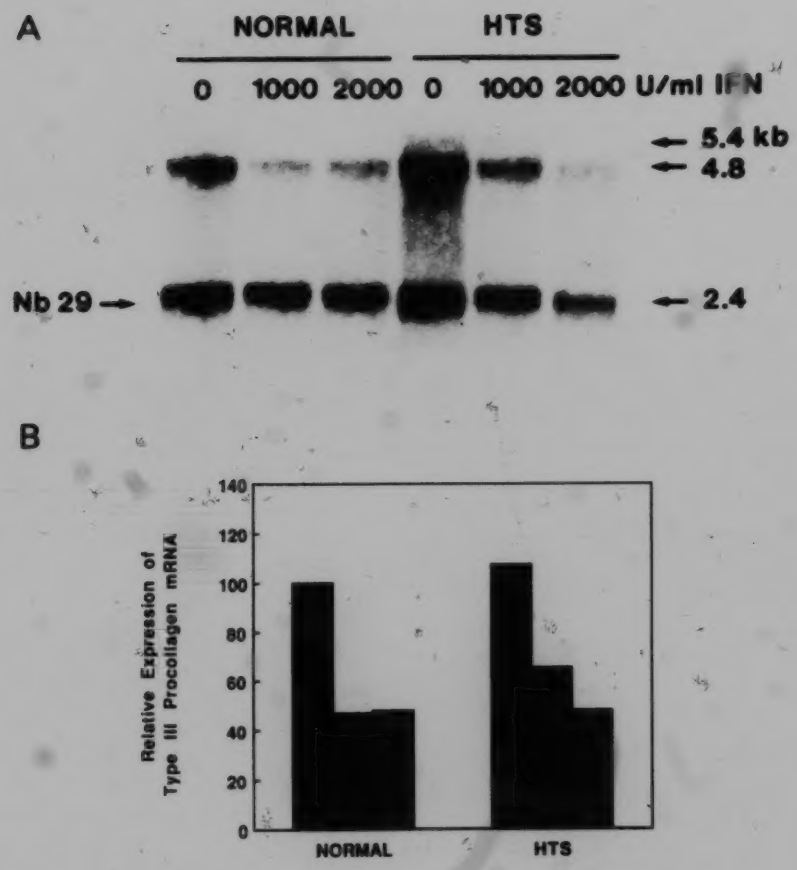

Figure 8. Effect of gamma IFN on type III procollagen mRNA expression in normal and hypertrophic scar fibroblasts from cell strain 2. Panel A shows the autoradiogram of the Northern blot of RNA from cells treated for 12 hours with varying doses of gamma IFN. Nb29 mRNA expression is included as a loading control. Relative expression of type III procollagen mRNA can be determined by quantitative densitometry after correcting for variation in loading (panel B); bars on the histogram represent cells receiving no treatment (■), 1000 U/mL gamma IFN (▨) or 2000 U/mL gamma IFN (■).

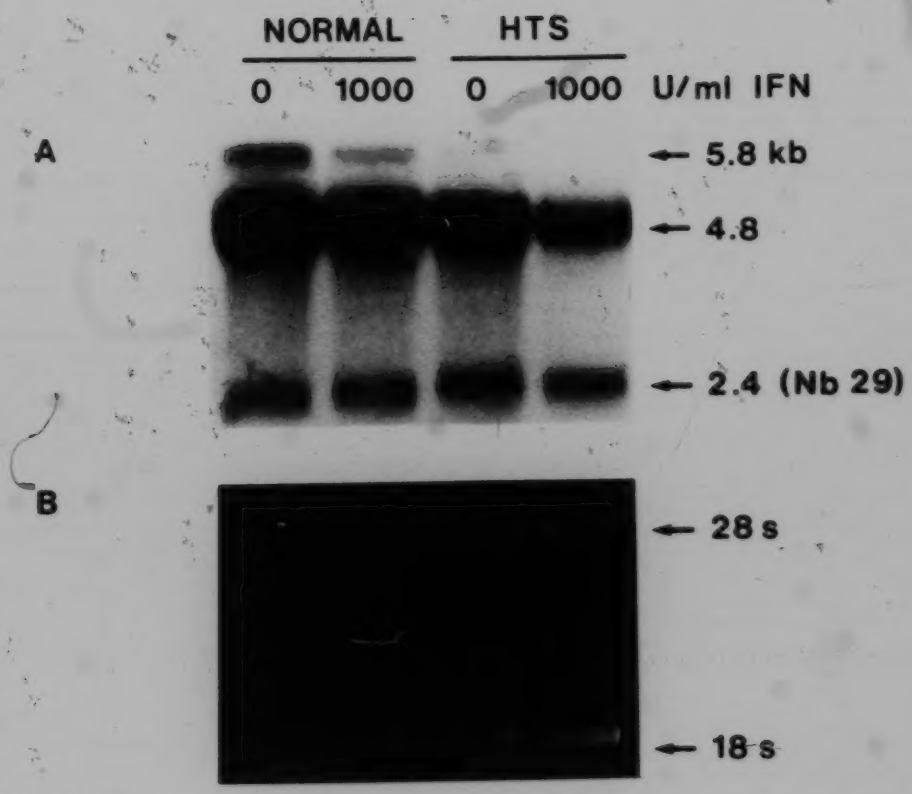

Figure 9. Effect of gamma IFN on Nb29 mRNA expression in cell strain 5. Panel A depicts the autoradiogram of the Northern blot of gamma IFN treated and untreated cells which has been hybridized with cDNA probes for type I procollagen mRNA and Nb29 mRNA. Panel B shows the corresponding ethidium bromide stained gel.

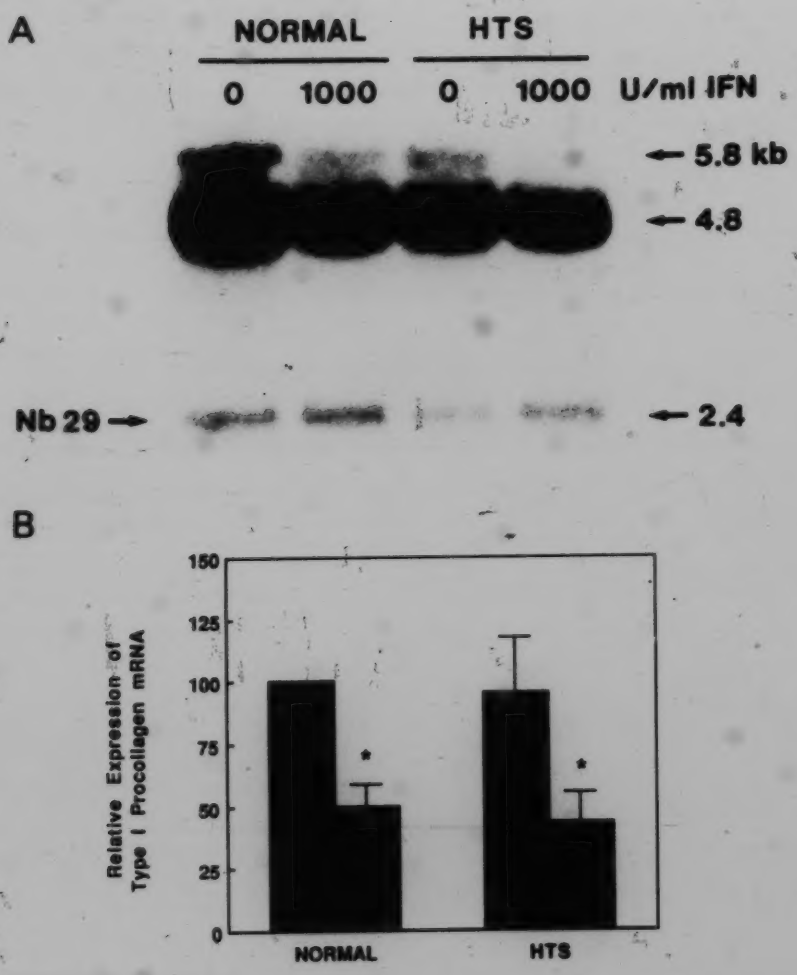

Figure 10. Effect of gamma IFN on Type I procollagen mRNA expression in fibroblasts from 5 paired normal/HTS cell strains. Panel A shows the autoradiogram of Northern analysis of pooled samples of RNA (2.5 ug of each pooled together) from cells treated (1000 U/mL gamma IFN) and untreated. Nb29 is included as a loading control. Panel B represents the combined densitometry results of individual Northern blots for each of the paired cell strains, comparing the effect of treatment with 1000 U/mL IFN (▨) to no treatment (■). Values represent means ± SEM (* $p<0.05$). For each strain, densities are relative to that of normal cells in the untreated state (for which there is thus no SEM).

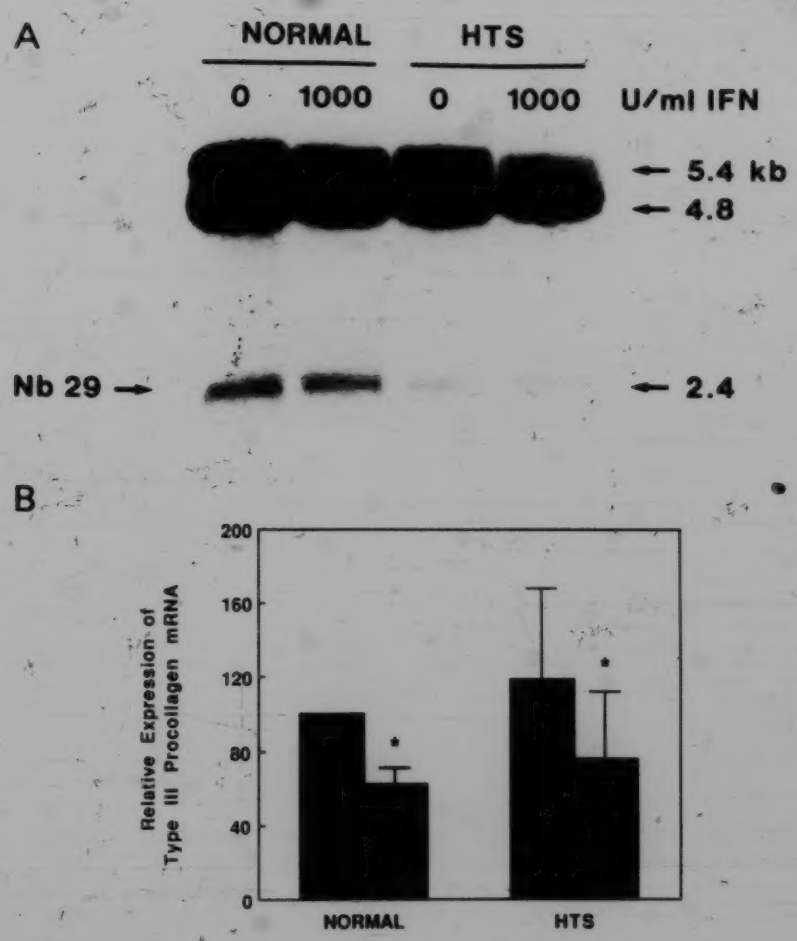

Figure 11. Effect of gamma IFN on Type III procollagen mRNA expression in fibroblasts from 5 paired normal/HTS cell strains. Panel A shows the autoradiogram of Northern analysis of pooled samples of RNA (2.5 ug of each pooled together) from cells treated (1000 U/mL gamma IFN) and untreated. Nb29 is included as a loading control. Panel B represents the combined results of individual Northern blots for each of the paired cell strains, comparing the effect of treatment with 1000 U/mL IFN (▨) to no treatment (■). Values represent means ± SEM (* $p<0.05$). For each strain, densities are relative to that of normal cells in the untreated state (for which there is thus no SEM).

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CHAPTER 6

DISCUSSION AND CONCLUSIONS

Ongoing improvement in the care of surgical patients has resulted in reduced mortality from traumatic injuries, including thermal injury. In the case of burn victims a natural consequence has been the emergence of hypertrophic scarring as a major form of morbidity. These patients often have extensive areas of injury which, should they become hypertrophic scars, may produce considerable functional limitation and unsitely physical appearance.

Current treatment of hypertrophic scarring includes mechanical pressure in the form of pressure garments and topical application of silicone gel sheets [1,2]. While these are safe and noninvasive treatments they are often inadequate for problematic scarring. Intralesional corticosteroid injections may dramatically improve isolated lesions however they may cause dermal thinning and epidermal atrophy. Furthermore, systemic side-effects make them impractical for cases of wide-spread hypertrophic scarring [3]. Surgical excision and resurfacing of the lesions is frequently complicated by recurrence [1]. While all of these treatment modalities have been clinically useful to some extent, the basis for their use is largely empirical and not founded on an understanding of the pathogenesis of hypertrophic scarring.

It is well known that during the usual process of wound healing, the initial burn (or other) injury is a stimulus to inflammation in which numerous inflammatory cells enter the wound. These in turn

release a number of angiogenic and fibroblast growth factors which stimulate the proliferation of fibroblasts and new blood vessels culminating in the laying down of collagen and other extracellular matrix components of scar tissue [4]. Hypertrophic scarring arises in healing wounds when there is an imbalance in the processes of collagen production and degradation, with the net result being overaccumulation of collagen within the wound [5]. The impetus for this thesis is the belief that this imbalance is at least in part due to an altered presence of various growth factors in the healing wound or an altered response of cells to these growth factors.

The objective of the present set of experiments was to study the effect of one growth factor, gamma interferon, on fibroblasts from hypertrophic scars using normal dermal fibroblasts from the same patients as controls. Gamma interferon is a cytokine produced by activated T lymphocytes and as such may play a role in regulation of wound healing. It has been shown to reduce collagen production by normal fibroblasts in vitro [6,7,8] and in vivo [9]. It was the hypothesis of these experiments that gamma interferon inhibits collagen production by hypertrophic scar fibroblasts. It was hoped that this information might not only improve understanding of the pathogenesis of hypertrophic scarring but also point the way to a new avenue of treatment, namely growth factor manipulation.

Our results suggest that gamma interferon is capable of inhibiting proliferation of HTS fibroblasts and of reducing collagen production by these cells. It also seems that the latter effect is at least in part a result of reduced expression of types I and III procollagen mRNA.

These findings lend support to the notion that gamma interferon might be useful as a therapeutic agent in the control of hypertrophic scarring. There have in fact been two small clinical studies in which intralesional administration of gamma IFN to patients with keloid or hypertrophic scarring was associated with some degree of clinical improvement [3,10]. It has also been administered systemically to patients with rheumatoid arthritis [11] and scleroderma [12], again with clinical improvement in these patients. Clinical studies with gamma IFN show it to be a well-tolerated drug. Reported side-effects of intralesional injection include headache, fever and myalgia, all of which are dose-dependent and are generally improved by analgesics or antipyretics [3]. Systemic administration has been infrequently associated with reversible granulocytopenia and elevation of hepatic enzymes [12]. Clinical improvement in a case of debilitating hypertrophic scarring would easily outweigh these side-effects.

The other issue that comes to light from these experiments is that of the pathogenesis of hypertrophic scarring. The important question exists as to why there is overaccumulation of collagen within hypertrophic scars. On one hand, the fibroblasts within these wounds may be intrinsically different from unaffected fibroblasts, being somehow changed or selected for by conditions within the healing wound. On the other hand, they may be very similar to normal fibroblasts but are responding to some ongoing signal or set of signals within the wound. These experiments, in which untreated normal and HTS cells under identical culturing condtions demonstrated similar characteristics and in which treated cells demonstrated the same responses to gamma IFN,

would argue for the latter hypothesis. Other investigators looking at collagen production by cultured normal and keloid fibroblasts have also shown no difference [13,14,15,16], albeit controls were not obtained from the same patients. Before accepting this hypothesis however, one should consider two other possibilities.

Firstly, the cell culture model chosen for these experiments has certain limitations. It is a useful system for studying isolated characteristics of cells and their response to isolated perturbations, such as the presence or absence of gamma interferon. The cell culture situation however, may not accurately reflect the in vitro situation. It has been shown by others that within the normal dermis of a given patient there exists heterogeneity amongst fibroblasts in their ability to synthesize collagen [17]. A culture of fibroblasts might not be representative of this heterogeneity. Furthermore, the process of culturing cells may in some way select for a cell type demonstrating certain characteristics. Bronson and colleagues have shown, for example, that cultured fibroblasts from normal skin produce different proportions of glycosaminoglycans than fibroblasts from the tissue itself [18]. The present studies did not look at the characteristics of fibroblasts in vivo.

Secondly, there exists the possibility that there are temporal differences in the behaviour of hypertrophic scar fibroblasts. As has been mentioned, the clinical process of hypertrophic scarring is a dynamic process beginning at the time of tissue injury with an inflammatory response which in turn gives way to a period of exuberant fibrosis and in most cases culminates in a state of quiescence. It is

possible that fibroblasts within the wound demonstrate changing behaviour during these different phases of clinical activity. These experiments did not examine fibroblasts from a given patient over the evolution of a hypertrophic scar.

Future work in this area should address the role of the wound milieu in the development of HTS, particularly the effect of various growth factors and solid-phase signals within the extracellular matrix including glycoproteins and proteoglycans. Transforming growth factor beta and interleukin 1, for example, are both known to enhance collagen synthesis by fibroblasts [8,19]. There are probably a number of such signals acting in concert to not only initiate the fibroproliferative response but also to terminate it. These studies would require a shift in focus from cells in culture to in situ techniques such as in situ hybridization to detect signals present in minute quantities or ex vivo techniques such as the polymerase chain reaction to amplify signals present in small quantities.

It would be of interest also to study changes in hypertrophic scar over the clinical course of the disease, looking at fibroblasts in culture as well as the various growth factor signals within the tissue itself.

Finally, given the current knowledge of the effect of gamma interferon on hypertrophic scar fibroblasts and the favorable experience with both local and systemic administration of gamma interferon in other fibrotic conditions, further efforts could be directed at clinical studies of the therapeutic effect of gamma interferon on problematic

hypertrophic scarring. It is likely that therapeutic manipulation of growth factors such as gamma interferon will prove useful clinically in the control of various disease states including hypertropic scarring.

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Appendix 1: Fibroblast Proliferation by Day 7 Amongst Individual Cell Strains After Treatment or No Treatment with IFN

Cell Count on Day 7

Cell Strain	Normal No IFN	Normal 1000 U/ml IFN	Normal 2000 U/ml IFN	HTS No IFN	HTS 1000 U/ml IFN	HTS 2000 U/ml IFN
1	26,823	9,306	6,588	31,368	15,988	10,853
2	26,550	16,103	12,580	14,783	7,177	6,637
3	37,190	30,007	28,773	24,070	16,400	15,017
4	73,108	42,841	39,311	84,083	32,031	28,738
5	50,181	41,733	42,671	58,181	32,058	28,896
Mean	42,770	27,998	25,985	42,497	20,731	18,028
S.E.M.	8,729	6,723	7,140	12,663	4,904	4,600

Appendix 2: Fibroblast Proliferation by Day 11 Amongst Individual Cell Strains After Treatment or No Treatment with IFN

Cell Count on Day 11

Cell Strain	Normal No IFN	Normal 1000 U/ml IFN	Normal 2000 U/ml IFN	HTS No IFN	HTS 1000 U/ml IFN	HTS 2000 U/ml IFN
1	56,472	28,074	15,169	117,229	54,602	46,087
2	81,510	36,567	29,140	56,133	17,620	13,583
3	92,000	77,213	63,036	69,249	43,600	43,724
4	182,374	121,599	105,957	174,992	93,344	83,702
5	110,748	93,418	84,988	134,698	69,526	65,221
Mean	104,621	71,374	59,658	110,460	55,738	50,463
S.E.M.	21,325	17,506	16,893	21,725	12,661	11,720

Appendix 3: Hydroxyproline Production By Individual Cell Strains After Treatment or No Treatment with IFN

Hydroxyproline Production (pg/cell/48 hours)

Cell Strain	Normal No IFN	Normal 1000 U/ml IFN	HTS No IFN	HTS 1000 U/ml IFN
1	8.81	7.63	3.59	2.35
2	4.47	2.69	2.30	1.85
3	4.65	4.04	2.91	1.93
4	4.57	2.82	4.39	2.28
5	6.22	4.55	5.46	3.98
Mean	5.74	4.35	3.73	2.48
S.E.M.	0.83	0.89	0.56	0.39

Appendix 4: Relative Expression of Type I Procollagen mRNA Amongst Individual Cell Strains

Relative Expression of Type I Procollagen mRNA

Cell Strain	Normal No IFN	Normal 1000 U/ml IFN	HTS No IFN	HTS 1000 U/ml IFN
1	100	50	178	89
2	100	31	93	32
3	100	31	75	33
4	100	82	86	40
5	100	54	45	22
Mean	100	49.6	95.4	43.2
S.E.M.	0	9.4	22.2	11.8

Appendix 5: Relative Expression of Type III Procollagen mRNA Amongst Individual Cell Strains

Relative Expression of Type III Procollagen mRNA

Cell Strain	Normal No IFN	Normal 1000 U/ml IFN	HTS No IFN	HTS 1000 U/ml IFN
1	100	47	308	214
2	100	46	107	79
3	100	55	65	42
4	100	97	82	31
5	100	64	33	14
Mean	100	61.8	119	76
S.E.M.	0	9.4	48.8	36.1